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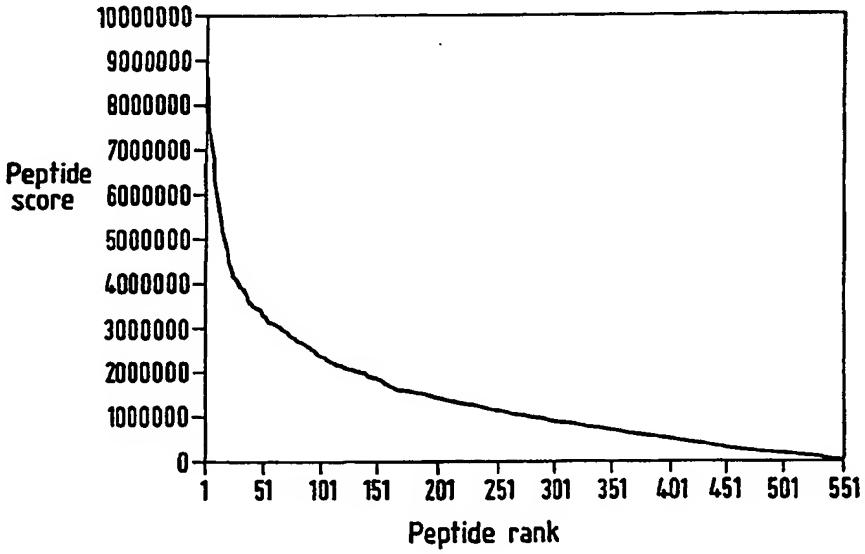

(57) Abstract

The invention provides a method for the prediction of the binding affinity of a peptide to a major histocompatilibity (MHC) class II molecules comprising; 1) ascertaining the characteristics of a MHC molecule binding groove, 2) presenting a selected peptide to the MHC molecule and ascertaining a first conformation score for each pocket bound peptide side–chain, 3) amending the conformation of each pocket bound peptide side–chain and ascertaining a second conformation score, 4) repeating step 3 with alternative conformations of each peptide pocket bound side–chain, 5) choosing the highest conformation score for each pocket bound peptide side–chain in each binding groove pockets, herein known as "the pocket", and 6) combining the highest conformation score for each pocket and ascertaining a binding score for the complete peptide.

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IDENTIFICATION OF MHC BINDING PEPTIDES

The present invention relates to a new method for the prediction of peptides which bind to major histocompatibility (MHC) class II molecules and to molecules created or modified through the use of these methods.

The immune system of the mammalian organism principally comprises two arms, the cellular immune system and the humoral or antibody-associated immune system. The cellular immune system is centred around the activity of T cells. There are two major classes of T cells, cytotoxic T lymphocytes (CTLs) which attack cells displaying foreign antigen complexed with MHC class I molecules, and helper T cells which react to cells displaying foreign antigens in a complex with MHC class II molecules resulting in the secretion of cytokines which can activate B cells to produce antibody molecules.

Humans express six different MHC class I genes and six different MHC class II genes, which are located on three highly polymorphic loci. This leads to considerable allelic variation in MHC molecules. The MHC class I consist of a α-chain and a β_2-microglobulin, the α-chain is split into three domains α_1, α_2 and α_3. α_1 and α_2 form the MHC class I binding groove which contains pockets that bind the side chains and the amino and carboxy termini of any peptide present in the groove. The MHC class II molecules comprise an α-chain and a β-chain, it is the α_1 and β_1 domains which create the MHC class II binding groove. The MHC class II binding groove also contains pockets but it does not bind the end termini of the peptide. For this reason the peptides bound by the MHC class II molecule can be longer and of a more variable length. The typical length of peptides complexed with a MHC class I or a MHC class II molecule are 8-10 amino acids and 13-20 amino acids, respectively.

At present only three MHC class II structure are available but

it is believed that the backbone structure of all MHC class II alleles presently identified are similar to that of HLA-DR1. Structures of different alleles can be predicted by using homology modelling. This involves identifying the amino acid differences near the binding groove and using a computer to change the conformation of the side-chains to give favourable steric and electrostatic arrangements and to make the pockets as large as possible. The end result is a three dimensional structure of a MHC class II molecule, which can be used in various experiments.

The ability to predict the peptides in a protein which can bind to a given MHC molecule has great value especially for medical applications. It is known, for example, that in certain auto-immune diseases, T cells react with self-peptides presented by MHC class II molecules. It would be valuable to predict which peptides from auto-immune proteins are presented by MHC class II molecules in these diseases as well as to predict the binding of analogues of these peptides synthesised as potential antagonists for the presentation of self-peptides. In the selection of peptides for synthetic vaccines, the ability to predict MHC class II binding peptides would be advantageous. In addition, where heterologous proteins are developed as medicines or diagnostic imaging agents, it would be advantageous to predict potential MHC class II binding peptides in order to eliminate these from the heterologous proteins before administration to patients.

While studies of peptides complexed with MHC class I molecules have revealed conserved "anchor" residues at certain positions within the presented peptides, such studies with peptides complexed with MHC class II molecules have been less successful mainly because of the greater length variability of such peptides and the consequent difficulty in aligning their sequences.

Methods for accurately predicting the binding potential of

peptides have been restricted to MHC class I interaction with a peptide. In one method using three-dimensional structures of MHC class I molecules, peptide binding is ranked in ascending order according to the energy values determined. This method requires that the MHC structure be known, or that there is an obvious molecular model for the MHC structure. An identical method is said to be available for MHC class II but it does not consider the longer average length of the peptide and the open-ended peptide binding groove of MHC class II molecules. Neither does it use the best potential conformation of peptide amino acid side-chains and, therefore the binding energies calculated are only approximations.

Another drawback of using the same method for MHC class I and MHC class II peptide binding is that the binding of peptides to MHC class II is less dependant on strict allele-specific binding motifs than peptides binding to MHC class I. Individual amino acids in the peptide play a more significant role in MHC class II binding than MHC class I such that the conformation of amino acid side-chains is proportionally more important to the accuracy of binding analysis. Therefore, known methods do not provide a general method for analysing the binding of peptides to three-dimensional structures of MHC class II. There is thus a need for improved methods for predicting the MHC class II binding potential of peptides.

An object of this invention is to provide a method for accurately predicting the binding affinity of a peptide fragment binding to a MHC class II molecule.

Another object of this invention is to provide a computer conditioned to perform the task of predicting the binding affinity of a peptide fragment binding to a MHC class II molecule.

A yet further object of this invention is to provide a vaccine derived from the peptide fragment whose binding affinity to

MHC class II molecules has been determined.

Another object of this invention is to provide a pharmaceutical composition which comprises a peptide whose binding affinity to MHC class II molecules has been determined.

According to the first aspect of this invention, there is provided a method for the prediction of the binding affinity of a peptide and a major histocompatibility (MHC) class II molecules comprising;

1) ascertaining the characteristics of a MHC molecule binding groove,

2) presenting a selected peptide to the MHC molecule and ascertaining a first conformation score for each pocket bound peptide side-chain,

3) amending the conformation of each pocket bound peptide side-chain and ascertaining a second conformation score,

4) repeating step 3 with alternative conformations of each peptide pocket bound side-chain,

5) choosing the highest conformation score for each pocket bound peptide side-chain,

6) combining the highest conformation score for each pocket-bound peptide side-chain and then ascertaining a binding score for the peptide.

It is particularly desirable to then compile information on all peptide fragments in a protein and compare the binding scores. It is preferable if the conformation of the backbone of the peptide fragment is also altered and the conformation score and the binding score is then reassessed.

The method of this invention thus involves assessing a binding score for all possible candidate peptides by considering the predicted three-dimensional conformations and interactions between the MHC and the peptide in the complex. The computed score indicates the predicted binding affinity for the

particular peptide binding with the MHC allele and can be used to predict whether the peptides are likely to bind, or not.

Preferably, the conformation score for each pocket bound peptide side-chain is ascertained by considering at least one of the following parameters:

a) the steric overlap between the pocket bound peptide residue bound in the pocket and an atom forming the pocket; this is value B,

b) the number of hydrogen bonds which can be formed between the pocket bound peptide residue and an atom forming the pocket; this is value C,

c) the strength of electrostatic interactions between any polar atoms of the pocket bound peptide residue and any polar atoms forming the pocket; this is value D, and

d) the number of favourable contacts between the pocket bound peptide residue and the MHC residues forming one of the pockets; this is value E.

The conformation score for each peptide is computed based upon the predicted atomic interactions between each of the pocket bound peptide residues and MHC pockets. The geometric constraints imposed on the peptide by the shape of the MHC binding groove play an important part of the scoring function. Favourable packing arrangements between peptide and MHC side-chains are rewarded by the scoring function, whilst arrangements involving steric overlap are penalised. Alternative conformation are tried for MHC residues if an MHC residue overlaps with a peptide side chain.

If no preferable conformation can be found the MHC side-chain is returned to its original conformation. In the event of more than a pocket residue side-chain overlapping with a pocket bound peptide side chain, the pocket residue side chains are adjusted in order of overlap severity, with the pocket residue side-chain which has the most severe overlap being adjusted first.

In preferred embodiments the steric overlap between the pocket bound peptide residue and the atoms forming the pocket can not be greater than 0.35 Angstroms, otherwise the residue is deemed unable to fit in the pocket.

Conveniently a favourable contact occurs when an atom from an MHC residue and an atom from the peptide residue have their centres separated by no more than the sum of their radii plus 0.5 Angstroms and are not overlapping.

Preferably the values B to E are imported into a first equation to give a conformation score(Z). The first equation is $Z_n=(cK_2C)-(cK_3D)+(cK_4E)-(cK_1B)$, where cK_1 to cK_4 are constants and n is the number of the pocket.

The value of cK_1 is between 50 and 150. Preferably between 75 and 125.

The value of cK_2 is between 1000 and 2000. Preferably between 1250 and 1750.

The value of cK_3 is between 250 and 750. Preferably between 350 and 650.

The value of cK_4 is between 500 and 1500. Preferably between 750 and 1250.

Conveniently the Z_n value for a pocket is multiplied by a coefficient, L, depending on the pockets importance in binding, to give a second Z_n value. The value L is in the range of 0.001 to 5. Larger pockets are considered more important in determining which peptide can bind, compared with the other smaller pockets, so the scores contributed by each pocket are weighted in proportion to the amount of the peptide side-chain buried by the surface of the MHC molecule. When binding to MHC class II molecules, peptides have shown high similarity in the degree to which their side-chains are buried

by the MHC surface, despite having dissimilar sequences.

Preferably all the Z_n values are summed to give a value J. Value J is the overall contributing score of all the pockets for a certain conformation of the peptide fragment.

Conveniently the MHC residue is paired with the pocket-bound peptide residue if an atom from the MHC residue and an atom from the pocket-bound peptide residue have their centres separated by no more than the sum of their van der Waal radii plus one Angstrom.

In a preferred embodiment a value A_n is calculated by summing the pairwise interaction frequencies of paired residues. As for the Z_n value, preferably the value A_n for a pocket is multiplied by a coefficient, X, depending on the pockets importance in binding. Preferably X is between 0.001 and 5.

Conveniently the A_n value for the pockets are summed to give a value P.

In a preferred embodiment the binding score is ascertained by at least one of the following parameters

a) the number of groove-bound hydrophobic residues; this is value F,

b) the number of non groove-bound hydrophilic residues; this is value G,

c) the number of peptide residues deemed to fit within their respective binding pocket; this is value H.

Preferably values F, G, H, J and P are imported into a second equation to give a first binding score, Y.

Conveniently the second equation is $Y=J*F^2*(G*H+1)+P$.

However, in the alternative, the term He, which evaluates the hydrophobicity of the pocket bound peptide side chains using

a hydrophobicity scale disclosed in Janin et al [1979] Nature, 277 pg 491, can also be used to determine the Y value. Accordingly, $Y=(bK_2C)-(bK_3D)+(bK_4E)-(bK_1B)+(bK_5He)+P$. The scale used in Janin et al to measure hydrophobicity has a range from -1.8 for lysine to 0.9 for cysteine.

It is known that peptides having favourable hydrophobic/hydrophobic interactions with solvent and MHC atoms have a higher binding affinity. Accordingly, it is preferable to include the term He.

The value of bK_1 is between 1 and 10. Preferably between 1 and 5.

The value of bK_2 is between 20 and 60. Preferably between 30 and 50.

The value of bK_3 is between 300 and 900. Preferably between 450 and 750.

The value of bK_4 is between 1 and 20. Preferably between 5 and 15.

The value of bK_5 is in between 1 and 800. Conveniently between 100 and 600. Preferably between 100 and 400.

In a preferred embodiment determination of the conformation score and the binding score are repeated for each pocket and each conformation of the peptide residue in said pocket. The conformation of the peptide is altered by rotating a side chain of the peptide residue by a pre-determined amount. In this way all possible conformations of the peptide side-chain in the pocket can be studied and the best or most likely conformation can be chosen to obtain the binding score.

The conformation of the backbone of the peptide fragment is changed by modelling the conformation of the backbone on any

one of 167 backbones which have been previously generated, based on human and murine crystallographic structures of MHC class II peptide complexes. The backbone conformation and the conformation of the peptide fragment side chains are altered systematically until the conformation score and the binding score of every possible conformation has been determined.

Conveniently the steps are repeated using different peptides from a protein.

In preferred embodiments the binding scores (Y) for different peptides are tabulated and compared. Peptides with the highest scores are predicted to have the highest binding affinity for the particular MHC allele.

In a preferred embodiment the method of determining the binding affinity of a peptide residue for an MHC class II molecule is used in the manufacture of a vaccine derived from a peptide identified by said method.

Preferably the method of determining the binding affinity of a peptide residue for an MHC class II molecule is used to remove potentially immunogenic sequences from a protein and thus reduce said proteins immunogenicity when administered to an organism.

Using the afore-detailed method it is possible to predict the peptides from an auto-immune protein which are presented by MHC class II molecules. Thereafter, it is possible to synthesise peptides which would be antagonists to the presentation of such peptides by the MHC class II molecules. It is also possible to determine any proteins in a vaccine containing heterologous proteins which might result in the stimulation of T cells due to their presentation on MHC class II molecules. These proteins could then be altered or removed depending on their function in the vaccine.

According to a second aspect of the invention there is provided a computer conditioned to receive information characterising a peptide bound to the MHC molecule and to utilise said information to perform a procedure having the following steps;

1) ascertaining the characteristics of a MHC molecule binding groove;

2) presenting a selected peptide, which is selected by a predetermined program, to the MHC molecule and ascertaining a first conformation score;

3) amending the conformation of the peptide, by way of a predetermined program, and ascertaining a second conformation score;

4) repeating step 3 with other conformations of the peptide;

5) selecting the peptide conformation with the highest conformation score; and

6) calculating the binding score from the conformation score.

Preferably the above detailed procedure also includes a step (7) which comprises repeating steps 1-4 with other peptide fragments in the protein to generate information on all peptide fragments in a protein so that a comparison can be made of the strength of the binding between the peptide and the MHC molecule.

Conveniently the above detailed procedure further comprising a step (8) which comprises altering the conformation of the backbone of the peptide fragment.

The use of a computer in such a task is important because there are hundreds of calculations to perform per peptide fragment. A computer conditioned to perform the task can systematically change the conformation of the side chains and the backbone of the peptide fragment while calculating the conformation score and the binding score.

According to a third aspect of the invention there is provided

a pharmaceutical composition made by determining the binding affinity of a peptide for a MHC class II molecule.

A pharmaceutical composition is thus engineered to contain a peptide which is presented by an MHC class II molecule and which therefore stimulates the bodies cellular immune system. Alternatively the pharmaceutical composition is engineered so that it does not include peptides which significantly stimulate the immune system.

The invention will now be described, by way of illustration only, with reference to the following examples, tables and figures accompanying the specification.

Figure 1 shows a graphical representation of the binding score distribution of all 554 13-mer Influenza haemagglutinin peptides bound to HLA-DRB1*0101.

Figure 2 shows a graphical representation of the binding score distribution of all 554 13-mer Influenza haemagglutinin peptides bound to HLA-DRB1*0401.

Table 1 shows the value for all the factors required to determine the binding score for the 15 peptides from Influenza haemagglutinin which have the highest binding affinity for HLA-DRB1*0101.

Table 2 shows the value for all the factors required to determine the binding score for the 15 peptides from Influenza haemagglutinin which have the highest binding affinity for HLA-DRB1*0401.

Table 3 lists the sequence difference between HLA-DRB1*0101 and HLA-DRB1*0401.

Table 4 shows the torsion angles of the mutated side chains in HLA-DRB1*0401.

Example 1

The following method was used to confirm that the peptide PKYVKQNTLKLAT, has a high affinity binding for the MHC molecule HLA-DRB1*0101.

The conformation score was calculated as follows for an oligomeric peptide having thirteen amino acid residues, herein known as a 13-mer peptide:

a) Calculate the steric overlap between the pocket bound peptide residue in the binding groove and an atom forming the pocket; this is value B.

b) Count the number of hydrogen bonds which could be formed between the pocket bound peptide residue and atoms forming the pocket; this is value C.

c) Calculate the strength of electrostatic interactions between any polar atoms of the pocket bound peptide residue and any polar atoms forming the pocket; this is value D.

d) Count the number of favourable contacts between the pocket bound peptide residue and atoms forming the pocket; this is value E.

These values were then transformed into a conformation score (Z) by using the following equation:

$$Z_n=(cK_2C)-(cK_3D)+(cK_4E)-(cK_1B)$$

where cK_1 to cK_4 are constants and n is the number of the pocket. CK_1, cK_2, cK_3 and cK_4 are equal to 100, 1500, 500 and 1000 respectively.

The conformation of each rotatable side chain of the pocket bound peptide bound residue was then altered by 30° and the conformation score was recalculated.

The above steps were repeated for each of the pockets and the

highest conformation score for each of the pockets was used to determine the binding score.

The binding score was determined by establishing values for the following parameters:

a) the number of groove-bound hydrophobic residues; this is value F.

b) the number of non groove-bound hydrophilic residues; this is value G.

c) the number of peptide residues deemed to fit within their respective binding groove; this is value H.

The conformational scores for pockets one and five were doubled and then all the conformational scores were summed to give a value J.

The above values were then imported in to the following equation in order to determine the binding score:

$$J*F^2*(G*H+1)+P$$

The binding scores for all the 13-mer peptides from Influenza Haemagglutinin binding with MHC molecule HLA-DRB1*0401 were calculated and the resultant top 15 binding scores are presented in Table 1. PKYVKQNTLKLAT has the 8th highest binding affinity for HLA-DRB1*0101 from all 554 possible overlapping 13-mer peptides.

Table 1

Rank	Seq.	Peptide	Binding Score	P	B	C	D	E	F	G	H
1	328	NTLKLATGMRNVP	9382500	15012	0.00	1		27	4	6	5
2	453	IDLTDSEMNKLFE	8288922	17964	0.72	1		40	3	6	5
3	373	NSEGTGQAADLKS	7520420	10661	0.68	0	+0.01	30	4	7	
4	504	HDVYRDEALNNRF	7211042	15527	0.56	1	-0.05	31	3	6	5
5	119	PDYASLRSLVASS	7174962	17351	0.68	1		40	4	4	5
6	461	NKLFEKTRRQLRE	7049469	19407	0.79	0	+0.01	56	2	7	5
7	122	ASLRSLVASSGTL	6922064	16346	0.09	0		25	4	4	5
8	322	PKYVKQNTLKLAT	6765975	18217	1.82	1		56	3	5	5
9	458	SEMNKLFEKTRRQ	6156822	16617	0.30	4	+0.08	44	2	7	5
10	513	NNRFQIKGVELKS	6096900	14052	1.32	3	-0.01	30	4	7	4
11	439	YNAELLVALENQH	5890199	14198	0.60	1		33	4	4	5
12	63	STGKICNNPHRIL	5887908	12776	0.75	5	-0.05	31	3	6	5
13	50	IEVTNATELVQSS	5503551	14297	0.95	2	+0.06	39	3	5	5
14	262	NSNGNLIAPRGYF	5284475	10102	0.09	1		21	4	5	5
15	257	DVLVINSNGNLIA	5239292	17028	1.35	2		35	3	4	5

Example 2

A method as described in Example 1 was used to confirm that the peptide PDYASLRSLVASS from Influenza haemagglutinin, has high affinity binding for the MHC molecule HLA-DRB1*0401.

The structure of HLA-DRB1*0401 is not known but a three dimensional model was constructed based on the known structure of HLA-DRB1*0101 by homology modelling. 10 amino acid differences between the two molecules were identified (see Table 2) and HLA-DRB1*0101 was mutated using the molecular modelling package 'Quanta' to produce a model of HLA-DRB1*0401.

Then the side-chain conformations of the 10 amino acids were adjusted interactively. In most cases, torsion angles were chosen which resulted in little or no steric overlap between the mutated residues and surrounding atoms. In the case of non-conserved residues which were either charged or whose side-chains were able to form hydrogen bonds, the potential to form favourable interactions was also considered. The placement of 13H, 28D and 71K was such that these residues were able to form a favourable electrostatic arrangement whilst at the same time, having minimum steric overlap with surrounding atoms. In the case of 30Y, this residue was positioned such that its hydroxyl group was situated close to the side-chain of 9E, where a hydrogen bond may be formed. The torsion angles chosen for the 10 mutated amino acid residues were calculated in accordance with the standard conventions and are listed in Table 3.

The binding scores for all 13-mer peptides from Influenza Haemagglutinin binding with MHC molecule HLA-DRB1*0401 were calculated and the resultant top 15 binding scores are presented in Table 4. PDYASLRSLVASS has the 9th highest binding affinity for HLA-DRB1*0401 from all 554 possible overlapping 13-mer peptides.

Table 2

Seq. Pos.	HLA-DRB1*0101	HLA-DRB1*0401
b9	Tryptophan	Glutamic acid
b11	Leucine	Valine
b13	Phenylalanine	Histidine
b26	Leucine	Phenylalanine
b28	Glutamic acid	Aspartic Acid
b30	Cysteine	Tyrosine
b31	Isoleucine	Phenylalanine
b33	Asparagine	Histidine
b37	Serine	Tyrosine
b71	Arginine	Lysine

Table 3

Residue	c1	c2	c3	c4
b9	-61°	-71°	-2°	
b11	168°			
b13	-38°	-63°		
b26	170°	57°		
b28	-174°	-15°		
b30	-174°	41°		
b31	-119°	-13°		
b33	-95°	-2°		
b37	-116°	-2°		
b71	-97°	-45°	172°	9°

Table 4

Rank	Seq.	Peptide	Binding Score	P	B	C	D	E	F	G	H
1	453	IDLTDSEMNKLFE	3070823	6559	0.36	0		42	3	6	5
2	373	NSEGTGQAADLKS	2988447	4182	0.36	0	+0.01	32	4	7	5
3	328	NTLKLATGMRNVP	2899375	4639	0.00	1		27	4	6	5
4	122	ASLRSLVASSGTL	2894599	6819	0.03	0		24	4	4	5
5	72	HRILDGIDCTLID	2820446	4623	0.60	1	+0.16	28	4	6	5
6	461	NKLFEKTRRQLRE	2662369	7203	0.36	0	-0.11	50	2	7	5
7	119	PDYASLRSLVASS	2616648	6184	0.11	1		32	4	4	5
8	188	DNFDKLYIWGIHH	2615259	5429	0.58	0		29	5	6	4
9	322	PKYVKQNTLKLAT	2515861	6407	0.46	2		44	3	5	5
10	232	NIGSRPWVRGLSS	2488137	4818	0.41	0	-0.02	35	4	5	5
11	504	HDVYRDEALNNRF	2353661	4965	0.05	1	-0.07	25	3	6	5
12	135	EFITEGFTWTGVT	2208179	3543	0.07	1		20	4	5	5
13	251	TIVKPGDVLVINS	2176819	5259	0.10	0		16	5	5	4
14	257	DVLVINSNGNLIA	2107570	6673	0.71	2		40	3	4	5
15	439	YNAELLVALENQH	2035430	4795	0.03	1		26	4	4	5

Example 3

A library of backbones were constructed by examining the crystal structure of the HLA-DR1 complexed with SEB super-antigen. This results in a collection of homogenous peptides within the MHC binding groove. The atomic positions of the peptide backbone, as shown in the PDB file produced from the crystal, were considered to be the `representative' backbone conformation of a peptide which binds to HLA-DR1.

Each of the peptide backbone conformations from the known MHC class II crystallographic structures are taken and after being transformed to the same frame of reference as the `representative' peptide had the differences between their Cα/Cβ positions and those of the `representative' peptide

calculated. These differences summarise the variability of Cα/Cβ atomic positions between the known peptides and the `representative' peptide.

The differences were doubled to take into account the fact that the variability of peptides thus far crystallised may not fully represent the true variability of peptides binding to MHC class II molecules. The differences were then used to define regions within which peptide Cα and Cβ atoms centres are constrained to lie.

An exhaustive search was then made through candidate peptide backbones. Starting from the `representative' peptide candidates are generated by adjusting backbone ϕ and ψ angles in ten degree steps from the N-terminus to the C-terminus. An adjustment was rejected if it led to any Cα or Cβ atom centre being outside the allowed region, derived above. An adjustment which did not violate the constraint results in a new backbone conformation which is stored within the peptide backbone library.

The x, y, and z co-ordinates of atoms in the backbones designated 0, 14, 62, 65, 75, 93, 104, 107, 112, 118, 129, 134, 141, 144 are given in Tables 5 to 18.

Table 5

Backbone 0					
Atom Number	Atom type	Position in peptide	x	y	z
0	N	0	19.913	86.191	20.687
1	CA	0	19.472	86.222	22.078
2	C	0	18.153	85.531	22.516
3	O	0	18.200	84.640	23.352
4	CB	0	19.504	87.660	22.593
5	N	1	16.984	85.957	22.044
6	CA	1	15.771	85.316	22.536
7	C	1	15.262	84.115	21.770
8	O	1	15.175	84.127	20.547
9	CB	1	14.663	86.325	22.743
10	N	2	14.959	83.055	22.510
11	CA	2	14.414	81.829	21.926
12	C	2	12.920	82.131	21.907
13	O	2	12.384	82.737	22.840
14	CB	2	14.756	80.548	22.811
15	N	3	12.283	81.841	20.784
16	CA	3	10.866	82.097	20.637
17	C	3	10.086	80.785	20.839
18	O	3	10.560	79.730	20.447
19	CB	3	10.624	82.744	19.230
20	N	4	8.951	80.855	21.528
21	CA	4	8.035	79.734	21.814
22	C	4	6.945	79.658	20.721
23	O	4	6.664	80.648	20.044
24	CB	4	7.330	79.991	23.185
25	N	5	6.355	78.499	20.461
26	CA	5	5.266	78.527	19.496
27	C	5	4.167	78.292	20.475
28	O	5	4.342	77.560	21.444
29	CB	5	5.349	77.437	18.471
30	N	6	3.044	78.938	20.261
31	CA	6	1.950	78.858	21.205
32	C	6	1.050	77.758	20.856
33	O	6	0.836	77.517	19.690
34	CB	6	1.163	80.226	21.247
35	N	7	0.420	77.190	21.863
36	CA	7	-0.503	76.102	21.660
37	C	7	-1.889	76.607	21.227
38	O	7	-2.429	77.551	21.833
39	CB	7	-0.611	75.340	22.937
40	N	8	-2.442	75.997	20.167
41	CA	8	-3.790	76.330	19.644

Table 5 continued

Atom Number	Atom type	Position in peptide	x	y	z
42	C	8	-4.839	75.618	20.504
43	O	8	-4.505	74.687	21.236
44	CB	8	-3.924	75.908	18.149
45	N	9	-6.093	76.041	20.436
46	CA	9	-7.113	75.382	21.236
47	C	9	-7.976	74.424	20.403
48	O	9	-8.366	74.742	19.266
49	CB	9	-7.963	76.413	21.973
50	N	10	-8.203	73.232	20.971
51	CA	10	-8.995	72.149	20.365
52	C	10	-10.492	72.527	20.200
53	O	10	-10.962	73.563	20.702
54	CB	10	-8.830	70.835	21.191
55	N	11	-11.238	71.661	19.523
56	CA	11	-12.654	71.907	19.270
57	C	11	-13.603	71.483	20.395
58	O	11	-13.661	70.302	20.800
59	CB	11	-13.072	71.269	17.940
60	N	12	-14.360	72.481	20.852
61	CA	12	-15.363	72.337	21.898
62	C	12	-14.758	72.166	23.281
63	O	12	-14.785	71.069	23.853
64	CB	12	-16.320	71.168	21.577

Table 6

Backbone 14					
Atom Number	Atom type	Position in peptide	x	y	z
0	N	0	0.000	0.000	0.000
1	CA	0	18.281	86.637	22.405
2	C	0	16.799	86.756	22.715
3	O	0	16.250	87.880	22.720
4	CB	0	0.000	0.000	0.000
5	N	1	16.174	85.601	22.931
6	CA	1	14.768	85.553	23.287
7	C	1	14.098	84.393	22.569
8	O	1	13.053	84.588	21.908
9	CB	1	14.090	86.846	22.869
10	N	2	14.723	83.223	22.680
11	CA	2	14.182	82.013	22.093
12	C	2	12.659	82.164	21.901
13	O	2	11.952	82.431	22.884
14	CB	2	14.470	80.825	22.994
15	N	3	12.242	82.022	20.649
16	CA	3	10.845	82.086	20.317
17	C	3	10.219	80.681	20.423
18	O	3	10.898	79.694	20.101
19	CB	3	10.669	82.621	18.906
20	N	4	8.980	80.660	20.898
21	CA	4	8.245	79.430	21.010
22	C	4	6.863	79.586	20.344
23	O	4	6.283	80.680	20.413
24	CB	4	8.071	79.059	22.472
25	N	5	6.427	78.504	19.710
26	CA	5	5.135	78.479	19.082
27	C	5	4.084	77.942	20.074
28	O	5	4.171	76.770	20.468
29	CB	5	5.174	77.593	17.848
30	N	6	3.174	78.832	20.452
31	CA	6	2.100	78.470	21.336
32	C	6	1.349	77.248	20.769
33	O	6	1.703	76.776	19.678
34	CB	6	1.139	79.635	21.492
35	N	7	0.381	76.781	21.550
36	CA	7	-0.441	75.677	21.137
37	C	7	-1.906	76.139	21.008
38	O	7	-2.505	76.533	22.020
39	CB	7	-0.346	74.551	22.153
40	N	8	-2.392	76.101	19.773
41	CA	8	-3.758	76.454	19.498
42	C	8	-4.704	75.537	20.299
43	O	8	-4.316	74.404	20.618
44	CB	8	-4.043	76.313	18.013

Table 6 continued

Atom Number	Atom type	Position in peptide	x	y	z
45	N	9	-5.873	76.084	20.610
46	CA	9	-6.881	75.338	21.313
47	C	9	-7.500	74.285	20.371
48	O	9	-7.243	74.336	19.159
49	CB	9	-7.964	76.275	21.818
50	N	10	-8.250	73.372	20.978
51	CA	10	-8.934	72.354	20.229
52	C	10	-10.393	72.786	19.976
53	O	10	-11.075	73.192	20.928
54	CB	10	-8.914	71.043	20.996
55	N	11	-10.781	72.710	18.708
56	CA	11	-12.127	73.032	18.320
57	C	11	-13.058	71.846	18.640
58	O	11	-13.254	70.984	17.770
59	CB	11	-12.180	73.341	16.834
60	N	12	-13.551	71.844	19.872
61	CA	12	-14.474	70.830	20.305
62	C	12	0.000	-12.127	73.032
63	O	12	18.356	0.000	-12.127
64	CB	12	0.000	0.000	0.000

Table 7

Backbone 62					
Atom Number	Atom type	Position in peptide	x	y	z
0	N	0	0.000	0.000	0.000
1	CA	0	18.315	86.971	22.396
2	C	0	16.796	86.979	22.404
3	O	0	16.173	87.867	21.780
4	CB	0	0.000	0.000	0.000
5	N	1	16.231	85.979	23.075
6	CA	1	14.791	85.876	23.216
7	C	1	14.286	84.665	22.451
8	O	1	13.659	84.820	21.380
9	CB	1	14.132	87.123	22.652
10	N	2	14.595	83.487	22.989
11	CA	2	14.144	82.241	22.404
12	C	2	12.614	82.280	22.212
13	O	2	11.890	82.495	23.195
14	CB	2	14.518	81.077	23.305
15	N	3	12.208	82.108	20.960
16	CA	3	10.810	82.071	20.629
17	C	3	10.289	80.623	20.734
18	O	3	11.105	79.691	20.783
19	CB	3	10.596	82.591	19.218
20	N	4	8.967	80.514	20.800
21	CA	4	8.328	79.228	20.852
22	C	4	6.861	79.356	20.395
23	O	4	6.157	80.256	20.876
24	CB	4	8.377	78.680	22.268
25	N	5	6.490	78.478	19.470
26	CA	5	5.140	78.440	18.978
27	C	5	4.171	78.141	20.139
28	O	5	4.543	77.392	21.055
29	CB	5	5.006	77.369	17.909
30	N	6	3.002	78.765	20.060
31	CA	6	1.975	78.549	21.042
32	C	6	1.039	77.416	20.577
33	O	6	1.276	76.842	19.503
34	CB	6	1.174	79.824	21.246
35	N	7	0.052	77.131	21.418
36	CA	7	-0.931	76.132	21.102
37	C	7	-2.325	76.784	21.008
38	O	7	-2.553	77.814	21.661
39	CB	7	-0.941	75.055	22.174
40	N	8	-3.166	76.177	20.179
41	CA	8	-4.518	76.638	20.020
42	C	8	-5.491	75.631	20.666
43	O	8	-5.155	74.441	20.754

Table 7 continued

Atom Number	Atom type	Position in peptide	x	y	z
44	CB	8	-4.845	76.793	18.545
45	N	9	-6.623	76.163	21.113
46	CA	9	-7.650	75.345	21.696
47	C	9	-8.161	74.329	20.655
48	O	9	-8.197	74.658	19.460
49	CB	9	-8.802	76.215	22.170
50	N	10	-8.492	73.143	21.153
51	CA	10	-9.030	72.107	20.315
52	C	10	-10.518	72.390	20.029
53	O	10	-11.258	72.730	20.964
54	CB	10	-8.887	70.758	21.000
55	N	11	-10.869	72.271	18.754
56	CA	11	-12.232	72.455	18.336
57	C	11	-13.047	71.182	18.641
58	O	11	-13.155	70.312	17.764
59	CB	11	-12.284	72.752	16.847
60	N	12	-13.544	71.124	19.871
61	CA	12	-14.366	70.022	20.291
62	C	12	0.000	-12.232	72.455
63	O	12	18.332	0.000	-12.232
64	CB	12	0.000	0.000	0.000

Table 8

Backbone 65					
Atom Number	Atom type	Position in peptide	x	y	z
0	N	0	0.000	0.000	0.000
1	CA	0	18.487	86.641	22.418
2	C	0	16.990	86.870	22.533
3	O	0	16.510	87.999	22.287
4	CB	0	0.000	0.000	0.000
5	N	1	16.279	85.796	22.868
6	CA	1	14.844	85.866	23.065
7	C	1	14.178	84.664	22.417
8	O	1	13.234	84.830	21.612
9	CB	1	14.301	87.132	22.424
10	N	2	14.699	83.484	22.746
11	CA	2	14.144	82.241	22.248
12	C	2	12.616	82.381	22.089
13	O	2	11.950	82.822	23.038
14	CB	2	14.457	81.109	23.212
15	N	3	12.150	82.035	20.895
16	CA	3	10.742	82.065	20.608
17	C	3	10.206	80.624	20.484
18	O	3	10.895	79.773	19.902
19	CB	3	10.491	82.818	19.314
20	N	4	9.029	80.419	21.065
21	CA	4	8.376	79.140	20.993
22	C	4	6.930	79.322	20.491
23	O	4	6.309	80.350	20.801
24	CB	4	8.365	78.486	22.364
25	N	5	6.484	78.339	19.718
26	CA	5	5.139	78.340	19.212
27	C	5	4.150	78.069	20.363
28	O	5	4.487	77.306	21.280
29	CB	5	4.985	77.274	18.142
30	N	6	3.002	78.731	20.275
31	CA	6	1.959	78.547	21.246
32	C	6	0.861	77.634	20.665
33	O	6	0.752	77.533	19.433
34	CB	6	1.360	79.890	21.628
35	N	7	0.134	76.994	21.573
36	CA	7	-0.959	76.143	21.187
37	C	7	-1.983	76.952	20.366
38	O	7	-1.708	78.116	20.039
39	CB	7	-1.631	75.569	22.422
40	N	8	-3.087	76.287	20.048
41	CA	8	-4.156	76.921	19.326
42	C	8	-5.496	76.242	19.676

Table 8 continued

Atom Number	Atom type	Position in peptide	x	y	z
43	O	8	-6.146	75.692	18.775
44	CB	8	-3.906	76.820	17.831
45	N	9	-5.817	76.283	20.964
46	CA	9	-7.058	75.736	21.439
47	C	9	-7.606	74.721	20.416
48	O	9	-7.311	74.855	19.219
49	CB	9	-8.071	76.849	21.649
50	N	10	-8.339	73.746	20.940
51	CA	10	-8.959	72.751	20.108
52	C	10	-10.421	73.147	19.824
53	O	10	-10.685	73.773	18.787
54	CB	10	-8.919	71.398	20.799
55	N	11	-11.294	72.734	20.735
56	CA	11	-12.689	73.067	20.635
57	C	11	-13.474	71.860	20.085
58	O	11	-13.031	71.253	19.099
59	CB	11	-12.873	74.262	19.715
60	N	12	-14.572	71.556	20.766
61	CA	12	-15.436	70.486	20.348
62	C	12	0.000	-12.689	73.067
63	O	12	18.675	0.000	-12.689
64	CB	12	0.000	0.000	0.000

Table 9

Backbone 75					
Atom Number	Atom type	Position in peptide	x	y	z
0	N	0	0.000	0.000	0.000
1	CA	0	18.442	86.539	22.377
2	C	0	16.947	86.419	22.136
3	O	0	16.452	86.839	21.066
4	CB	0	0.000	0.000	0.000
5	N	1	16.265	85.822	23.109
6	CA	1	14.823	85.676	23.048
7	C	1	14.466	84.417	22.277
8	O	1	14.197	84.487	21.057
9	CB	1	14.218	86.875	22.338
10	N	2	14.505	83.290	22.985
11	CA	2	14.144	82.013	22.404
12	C	2	12.615	81.942	22.214
13	O	2	11.895	81.727	23.200
14	CB	2	14.601	80.882	23.308
15	N	3	12.201	82.159	20.971
16	CA	3	10.808	82.078	20.626
17	C	3	10.331	80.615	20.726
18	O	3	11.176	79.709	20.772
19	CB	3	10.592	82.592	19.213
20	N	4	9.013	80.465	20.789
21	CA	4	8.414	79.160	20.836
22	C	4	6.944	79.245	20.377
23	O	4	6.322	80.304	20.544
24	CB	4	8.478	78.609	22.251
25	N	5	6.482	78.145	19.793
26	CA	5	5.116	78.053	19.354
27	C	5	4.181	77.969	20.577
28	O	5	4.609	77.470	21.629
29	CB	5	4.932	76.823	18.483
30	N	6	2.974	78.490	20.389
31	CA	6	1.974	78.445	21.420
32	C	6	0.736	77.679	20.910
33	O	6	0.349	77.867	19.748
34	CB	6	1.576	79.855	21.821
35	N	7	0.206	76.836	21.788
36	CA	7	-0.980	76.086	21.478
37	C	7	-1.844	76.872	20.470
38	O	7	-1.448	77.977	20.071
39	CB	7	-1.778	75.828	22.745
40	N	8	-2.952	76.249	20.088
41	CA	8	-3.885	76.873	19.189

Table 9 continued

Atom Number	Atom type	Position in peptide	x	y	z
42	C	8	-5.324	76.483	19.579
43	O	8	-6.195	76.435	18.698
44	CB	8	-3.604	76.435	17.762
45	N	9	-5.491	76.194	20.865
46	CA	9	-6.786	75.859	21.391
47	C	9	-7.424	74.747	20.535
48	O	9	-7.209	74.729	19.314
49	CB	9	-7.681	77.087	21.388
50	N	10	-8.142	73.864	21.219
51	CA	10	-8.840	72.797	20.556
52	C	10	-10.312	73.196	20.334
53	O	10	-10.616	73.833	19.314
54	CB	10	-8.772	71.532	21.394
55	N	11	-11.149	72.774	21.275
56	CA	11	-12.546	73.108	21.233
57	C	11	-13.321	72.011	20.475
58	O	11	-12.815	71.509	19.460
59	CB	11	-12.741	74.445	20.540
60	N	12	-14.483	71.674	21.023
61	CA	12	-15.343	70.702	20.406
62	C	12	0.000	-12.546	73.108
63	O	12	18.817	0.000	-12.546
64	CB	12	0.000	0.000	0.000

Table 10

Backbone 93					
Atom Number	Atom type	Position in peptide	x	y	z
0	N	0	0.000	0.000	0.000
1	CA	0	18.249	86.312	21.629
2	C	0	16.910	86.341	22.345
3	O	0	16.646	87.271	23.139
4	CB	0	0.000	0.000	0.000
5	N	1	16.080	85.351	22.027
6	CA	1	14.782	85.213	22.662
7	C	1	14.078	83.978	22.127
8	O	1	12.999	84.095	21.505
9	CB	1	13.932	86.434	22.357
10	N	2	14.712	82.828	22.345
11	CA	2	14.144	81.558	21.938
12	C	2	12.613	81.689	21.812
13	O	2	11.912	81.568	22.828
14	CB	2	14.484	80.486	22.959
15	N	3	12.179	81.964	20.587
16	CA	3	10.775	82.068	20.300
17	C	3	10.163	80.658	20.176
18	O	3	10.712	79.826	19.439
19	CB	3	10.564	82.834	19.005
20	N	4	9.085	80.454	20.925
21	CA	4	8.374	79.206	20.882
22	C	4	7.026	79.401	20.159
23	O	4	6.568	80.546	20.036
24	CB	4	8.130	78.697	22.292
25	N	5	6.482	78.283	19.690
26	CA	5	5.203	78.295	19.035
27	C	5	4.087	78.033	20.066
28	O	5	4.298	77.235	20.991
29	CB	5	5.163	77.229	17.954
30	N	6	2.980	78.741	19.876
31	CA	6	1.833	78.572	20.726
32	C	6	1.164	77.213	20.434
33	O	6	1.603	76.513	19.510
34	CB	6	0.839	79.695	20.486
35	N	7	0.169	76.899	21.254
36	CA	7	-0.585	75.687	21.080
37	C	7	-2.092	76.013	21.037
38	O	7	-2.667	76.338	22.086
39	CB	7	-0.300	74.729	22.223
40	N	8	-2.639	75.944	19.829
41	CA	8	-4.045	76.173	19.635
42	C	8	-4.853	75.344	20.653
43	O	8	-4.314	74.368	21.198

Table 10 continued

Atom Number	Atom type	Position in peptide	x	y	z
44	CB	8	-4.445	75.782	18.223
45	N	9	-6.082	75.791	20.882
46	CA	9	-6.974	75.097	21.769
47	C	9	-8.018	74.312	20.948
48	O	9	-8.754	74.928	20.163
49	CB	9	-7.679	76.089	22.679
50	N	10	-8.002	72.999	21.144
51	CA	10	-8.947	72.137	20.488
52	C	10	-10.274	72.891	20.269
53	O	10	-10.348	73.727	19.356
54	CB	10	-9.194	70.899	21.332
55	N	11	-11.256	72.533	21.087
56	CA	11	-12.539	73.179	21.038
57	C	11	-13.542	72.288	20.278
58	O	11	-13.224	71.836	19.167
59	CB	11	-12.418	74.524	20.343
60	N	12	-14.678	72.054	20.925
61	CA	12	-15.731	71.281	20.326
62	C	12	0.000	-12.539	73.179
63	O	12	18.616	0.000	-12.539
64	CB	12	0.000	0.000	0.000

Table 11

Backbone 104					
Atom Number	Atom type	Position in peptide	x	y	z
0	N	0	0.000	0.000	0.000
1	CA	0	18.400	86.585	22.355
2	C	0	16.914	86.850	22.523
3	O	0	16.453	87.991	22.296
4	CB	0	0.000	0.000	0.000
5	N	1	16.189	85.793	22.880
6	CA	1	14.763	85.897	23.128
7	C	1	14.059	84.662	22.593
8	O	1	12.980	84.778	21.971
9	CB	1	14.210	87.122	22.421
10	N	2	14.693	83.511	22.810
11	CA	2	14.125	82.241	22.404
12	C	2	12.594	82.372	22.277
13	O	2	11.945	82.807	23.241
14	CB	2	14.465	81.169	23.424
15	N	3	12.104	82.026	21.093
16	CA	3	10.690	82.048	20.837
17	C	3	10.159	80.604	20.723
18	O	3	10.919	79.713	20.317
19	CB	3	10.406	82.801	19.548
20	N	4	8.902	80.444	21.120
21	CA	4	8.250	79.166	21.029
22	C	4	6.905	79.319	20.290
23	O	4	6.415	80.450	20.160
24	CB	4	8.009	78.605	22.420
25	N	5	6.401	78.185	19.817
26	CA	5	5.130	78.158	19.147
27	C	5	4.011	77.862	20.165
28	O	5	4.164	76.935	20.975
29	CB	5	5.135	77.091	18.066
30	N	6	2.968	78.680	20.096
31	CA	6	1.823	78.502	20.947
32	C	6	1.166	77.138	20.656
33	O	6	1.718	76.360	19.864
34	CB	6	0.819	79.617	20.708
35	N	7	0.047	76.906	21.334
36	CA	7	-0.707	75.699	21.135
37	C	7	-2.213	76.030	21.083
38	O	7	-2.793	76.357	22.129
39	CB	7	-0.435	74.724	22.267
40	N	8	-2.754	75.961	19.873
41	CA	8	-4.157	76.194	19.670
42	C	8	-4.974	75.368	20.684
43	O	8	-4.444	74.387	21.228

Table 11 continued

Atom Number	Atom type	Position in peptide	x	y	z
44	CB	8	-4.550	75.803	18.256
45	N	9	-6.200	75.824	20.911
46	CA	9	-7.100	75.134	21.794
47	C	9	-8.146	74.358	20.969
48	O	9	-8.997	74.991	20.328
49	CB	9	-7.800	76.129	22.704
50	N	10	-8.007	73.038	21.000
51	CA	10	-8.934	72.175	20.320
52	C	10	-10.266	72.919	20.092
53	O	10	-10.341	73.752	19.177
54	CB	10	-9.181	70.924	21.145
55	N	11	-11.249	72.557	20.907
56	CA	11	-12.537	73.194	20.850
57	C	11	-13.529	72.294	20.086
58	O	11	-13.514	72.297	18.847
59	CB	11	-12.421	74.537	20.152
60	N	12	-14.310	71.549	20.860
61	CA	12	-15.320	70.695	20.297
62	C	12	0.000	-12.537	73.194
63	O	12	18.422	0.000	-12.537
64	CB	12	0.000	0.000	0.000

Table 12

Backbone 107					
Atom Number	Atom type	Position in peptide	x	y	z
0	N	0	0.000	0.000	0.000
1	CA	0	18.468	86.641	22.418
2	C	0	16.971	86.870	22.533
3	O	0	16.491	87.999	22.287
4	CB	0	0.000	0.000	0.000
5	N	1	16.260	85.796	22.868
6	CA	1	14.825	85.866	23.065
7	C	1	14.159	84.664	22.417
8	O	1	13.215	84.830	21.612
9	CB	1	14.282	87.132	22.424
10	N	2	14.680	83.484	22.746
11	CA	2	14.125	82.241	22.248
12	C	2	12.597	82.381	22.089
13	O	2	11.931	82.822	23.038
14	CB	2	14.438	81.109	23.212
15	N	3	12.131	82.035	20.895
16	CA	3	10.723	82.065	20.608
17	C	3	10.187	80.624	20.484
18	O	3	10.876	79.773	19.902
19	CB	3	10.472	82.818	19.314
20	N	4	9.010	80.419	21.065
21	CA	4	8.357	79.140	20.993
22	C	4	6.911	79.322	20.491
23	O	4	6.290	80.350	20.801
24	CB	4	8.346	78.486	22.364
25	N	5	6.465	78.339	19.718
26	CA	5	5.120	78.340	19.212
27	C	5	4.131	78.069	20.363
28	O	5	4.469	77.306	21.280
29	CB	5	4.966	77.274	18.142
30	N	6	2.983	78.731	20.275
31	CA	6	1.940	78.547	21.246
32	C	6	0.842	77.634	20.665
33	O	6	0.733	77.533	19.433
34	CB	6	1.341	79.890	21.628
35	N	7	0.115	76.994	21.573
36	CA	7	-0.978	76.143	21.187
37	C	7	-2.002	76.952	20.366
38	O	7	-1.726	78.116	20.039
39	CB	7	-1.650	75.569	22.422
40	N	8	-3.106	76.287	20.048
41	CA	8	-4.175	76.921	19.326
42	C	8	-5.514	76.242	19.676
43	O	8	-6.165	75.692	18.775

Table 12 continued

Atom Number	Atom type	Position in peptide	x	y	z
44	CB	8	-3.925	76.820	17.831
45	N	9	-5.836	76.283	20.964
46	CA	9	-7.077	75.736	21.439
47	C	9	-7.625	74.721	20.416
48	O	9	-7.330	74.855	19.219
49	CB	9	-8.090	76.849	21.649
50	N	10	-8.358	73.746	20.940
51	CA	10	-8.977	72.751	20.108
52	C	10	-10.440	73.147	19.824
53	O	10	-10.703	73.773	18.787
54	CB	10	-8.938	71.398	20.799
55	N	11	-11.313	72.734	20.735
56	CA	11	-12.708	73.067	20.635
57	C	11	-13.493	71.860	20.085
58	O	11	-13.050	71.253	19.099
59	CB	11	-12.892	74.262	19.715
60	N	12	-14.591	71.556	20.766
61	CA	12	-15.455	70.486	20.348
62	C	12	0.000	-12.708	73.067
63	O	12	18.675	0.000	-12.708
64	CB	12	0.000	0.000	0.000

Table 13

Backbone 112					
Atom Number	Atom type	Position in peptide	x	y	z
0	N	0	0.000	0.000	0.000
1	CA	0	18.408	86.726	22.399
2	C	0	16.919	86.606	22.121
3	O	0	16.449	87.028	21.041
4	CB	0	0.000	0.000	0.000
5	N	1	16.215	86.005	23.077
6	CA	1	14.774	85.858	22.981
7	C	1	14.438	84.649	22.125
8	O	1	14.190	84.795	20.907
9	CB	1	14.176	87.097	22.337
10	N	2	14.470	83.480	22.761
11	CA	2	14.125	82.241	22.093
12	C	2	12.600	82.176	21.872
13	O	2	11.849	82.152	22.858
14	CB	2	14.572	81.057	22.932
15	N	3	12.224	82.187	20.598
16	CA	3	10.839	82.083	20.230
17	C	3	10.319	80.669	20.557
18	O	3	11.133	79.744	20.692
19	CB	3	10.674	82.359	18.745
20	N	4	9.001	80.583	20.701
21	CA	4	8.361	79.323	20.960
22	C	4	6.868	79.411	20.585
23	O	4	6.126	80.158	21.239
24	CB	4	8.500	78.961	22.429
25	N	5	6.516	78.676	19.537
26	CA	5	5.150	78.615	19.095
27	C	5	4.229	78.301	20.291
28	O	5	4.706	77.734	21.285
29	CB	5	4.995	77.540	18.033
30	N	6	2.976	78.716	20.149
31	CA	6	1.986	78.455	21.158
32	C	6	0.948	77.449	20.621
33	O	6	1.060	77.031	19.459
34	CB	6	1.291	79.747	21.552
35	N	7	0.020	77.088	21.499
36	CA	7	-1.045	76.194	21.133
37	C	7	-2.219	76.999	20.540
38	O	7	-2.062	78.205	20.301
39	CB	7	-1.517	75.422	22.353
40	N	8	-3.314	76.286	20.301
41	CA	8	-4.508	76.904	19.793
42	C	8	-5.720	75.987	20.056
43	O	8	-5.881	74.984	19.345
44	CB	8	-4.369	77.156	18.302
45	N	9	-6.483	76.357	21.078

Table 13 continued

Atom Number	Atom type	Position in peptide	x	y	z
46	CA	9	-7.676	75.631	21.417
47	C	9	-7.858	74.446	20.447
48	O	9	-7.297	74.482	19.341
49	CB	9	-8.883	76.549	21.338
50	N	10	-8.598	73.451	20.920
51	CA	10	-8.898	72.298	20.116
52	C	10	-10.415	72.236	19.842
53	O	10	-11.204	72.400	20.784
54	CB	10	-8.455	71.034	20.832
55	N	11	-10.740	72.040	18.569
56	CA	11	-12.112	71.910	18.163
57	C	11	-12.689	70.583	18.695
58	O	11	-12.384	69.523	18.128
59	CB	11	-12.211	71.942	16.648
60	N	12	-13.459	70.705	19.770
61	CA	12	-14.109	69.563	20.354
62	C	12	0.000	-12.112	71.910
63	O	12	18.708	0.000	-12.112
64	CB	12	0.000	0.000	0.000

Table 14

Backbone 118					
Atom Number	Atom type	Position in peptide	x	y	z
0	N	0	0.000	0.000	0.000
1	CA	0	18.471	86.536	22.407
2	C	0	16.968	86.701	22.266
3	O	0	16.498	87.742	21.755
4	CB	0	0.000	0.000	0.000
5	N	1	16.246	85.665	22.686
6	CA	1	14.795	85.690	22.663
7	C	1	14.271	84.435	21.986
8	O	1	13.620	84.525	20.922
9	CB	1	14.318	86.904	21.884
10	N	2	14.591	83.292	22.589
11	CA	2	14.125	82.013	22.093
12	C	2	12.591	82.045	21.934
13	O	2	11.881	82.067	22.951
14	CB	2	14.518	80.907	23.057
15	N	3	12.165	82.081	20.677
16	CA	3	10.762	82.064	20.366
17	C	3	10.221	80.625	20.479
18	O	3	11.005	79.674	20.343
19	CB	3	10.536	82.588	18.958
20	N	4	8.925	80.541	20.756
21	CA	4	8.263	79.268	20.845
22	C	4	6.879	79.352	20.171
23	O	4	6.325	80.457	20.070
24	CB	4	8.101	78.868	22.301
25	N	5	6.413	78.195	19.716
26	CA	5	5.115	78.103	19.106
27	C	5	4.061	77.755	20.177
28	O	5	4.217	76.737	20.866
29	CB	5	5.122	77.034	18.027
30	N	6	3.069	78.632	20.282
31	CA	6	1.984	78.421	21.202
32	C	6	1.060	77.308	20.670
33	O	6	1.327	76.771	19.584
34	CB	6	1.192	79.706	21.374
35	N	7	0.048	76.997	21.472
36	CA	7	-0.928	76.012	21.093
37	C	7	-2.316	76.673	20.976
38	O	7	-2.546	77.708	21.619
39	CB	7	-0.975	74.902	22.128
40	N	8	-3.150	76.066	20.139
41	CA	8	-4.496	76.535	19.959
42	C	8	-5.484	75.538	20.596
43	O	8	-5.163	74.343	20.680
44	CB	8	-4.801	76.684	18.479

Table 14 continued

Atom Number	Atom type	Position in peptide	x	y	z
45	N	9	-6.612	76.081	21.040
46	CA	9	-7.652	75.273	21.615
47	C	9	-8.169	74.268	20.567
48	O	9	-8.200	74.604	19.374
49	CB	9	-8.795	76.156	22.087
50	N	10	-8.513	73.083	21.059
51	CA	10	-9.059	72.056	20.214
52	C	10	-10.544	72.355	19.925
53	O	10	-11.281	72.703	20.859
54	CB	10	-8.931	70.703	20.892
55	N	11	-10.894	72.239	18.649
56	CA	11	-12.254	72.439	18.229
57	C	11	-13.135	71.287	18.754
58	O	11	-13.091	70.187	18.183
59	CB	11	-12.328	72.490	16.713
60	N	12	-13.856	71.586	19.828
61	CA	12	-14.763	70.632	20.406
62	C	12	0.000	-12.254	72.439
63	O	12	18.754	0.000	-12.254
64	CB	12	0.000	0.000	0.000

Table 15

Backbone 129					
Atom Number	Atom type	Position in peptide	x	y	z
0	N	0	0.000	0.000	0.000
1	CA	0	18.495	86.291	22.091
2	C	0	17.099	86.364	22.686
3	O	0	16.668	87.449	23.137
4	CB	0	0.000	0.000	0.000
5	N	1	16.409	85.228	22.645
6	CA	1	15.079	85.125	23.217
7	C	1	14.331	83.972	22.570
8	O	1	13.400	84.204	21.766
9	CB	1	14.313	86.412	22.964
10	N	2	14.767	82.758	22.900
11	CA	2	14.125	81.558	22.404
12	C	2	12.611	81.805	22.245
13	O	2	11.911	81.927	23.261
14	CB	2	14.358	80.407	23.367
15	N	3	12.194	81.901	20.988
16	CA	3	10.803	82.082	20.676
17	C	3	10.173	80.727	20.297
18	O	3	10.650	80.085	19.349
19	CB	3	10.652	83.058	19.522
20	N	4	9.165	80.348	21.074
21	CA	4	8.445	79.131	20.819
22	C	4	7.047	79.462	20.257
23	O	4	6.608	80.615	20.376
24	CB	4	8.305	78.330	22.102
25	N	5	6.442	78.450	19.647
26	CA	5	5.114	78.588	19.113
27	C	5	4.079	78.178	20.180
28	O	5	4.373	77.289	20.993
29	CB	5	4.955	77.714	17.881
30	N	6	2.945	78.866	20.145
31	CA	6	1.864	78.568	21.044
32	C	6	1.193	77.243	20.630
33	O	6	1.658	76.606	19.673
34	CB	6	0.841	79.690	21.018
35	N	7	0.165	76.881	21.388
36	CA	7	-0.594	75.695	21.099
37	C	7	-2.093	76.044	21.014
38	O	7	-2.691	76.384	22.046
39	CB	7	-0.369	74.657	22.184
40	N	8	-2.610	75.977	19.793
41	CA	8	-4.006	76.226	19.560
42	C	8	-4.854	75.414	20.559
43	O	8	-4.305	74.533	21.237
44	CB	8	-4.374	75.835	18.139
45	N	9	-6.130	75.774	20.624
46	CA	9	-7.058	75.079	21.473
47	C	9	-8.093	74.330	20.610

Table 15 continued

Atom Number	Atom type	Position in peptide	x	y	z
48	O	9	-8.797	74.974	19.819
49	CB	9	-7.768	76.066	22.384
50	N	10	-8.107	73.013	20.781
51	CA	10	-9.049	72.181	20.083
52	C	10	-10.358	72.962	19.848
53	O	10	-10.355	73.921	19.062
54	CB	10	-9.337	70.929	20.893
55	N	11	-11.409	72.493	20.510
56	CA	11	-12.689	73.142	20.432
57	C	11	-13.742	72.155	19.889
58	O	11	-13.537	71.595	18.802
59	CB	11	-12.603	74.353	19.519
60	N	12	-14.788	71.968	20.684
61	CA	12	-15.877	71.114	20.295
62	C	12	0.000	-12.689	73.142
63	O	12	18.488	0.000	-12.689
64	CB	12	0.000	0.000	0.000

Table 16

Backbone 134					
Atom Number	Atom type	Position in peptide	x	y	z
0	N	0	0.000	0.000	0.000
1	CA	0	18.230	86.312	21.629
2	C	0	16.891	86.341	22.345
3	O	0	16.627	87.271	23.139
4	CB	0	0.000	0.000	0.000
5	N	1	16.061	85.351	22.027
6	CA	1	14.763	85.213	22.662
7	C	1	14.059	83.978	22.127
8	O	1	12.980	84.095	21.505
9	CB	1	13.913	86.434	22.357
10	N	2	14.693	82.828	22.345
11	CA	2	14.125	81.558	21.938
12	C	2	12.594	81.689	21.812
13	O	2	11.893	81.568	22.828
14	CB	2	14.465	80.486	22.959
15	N	3	12.160	81.964	20.587
16	CA	3	10.756	82.068	20.300
17	C	3	10.144	80.658	20.176
18	O	3	10.693	79.826	19.439
19	CB	3	10.545	82.834	19.005
20	N	4	9.066	80.454	20.925
21	CA	4	8.355	79.206	20.882
22	C	4	7.007	79.401	20.159
23	O	4	6.549	80.546	20.036
24	CB	4	8.111	78.697	22.292
25	N	5	6.463	78.283	19.690
26	CA	5	5.184	78.295	19.035
27	C	5	4.068	78.033	20.066
28	O	5	4.279	77.235	20.991
29	CB	5	5.144	77.229	17.954
30	N	6	2.961	78.741	19.876
31	CA	6	1.814	78.572	20.726
32	C	6	1.146	77.213	20.434
33	O	6	1.584	76.513	19.510
34	CB	6	0.820	79.695	20.486
35	N	7	0.150	76.899	21.254
36	CA	7	-0.604	75.687	21.080
37	C	7	-2.110	76.013	21.037
38	O	7	-2.686	76.338	22.086
39	CB	7	-0.319	74.729	22.223
40	N	8	-2.658	75.944	19.829
41	CA	8	-4.064	76.173	19.635
42	C	8	-4.872	75.344	20.653
43	O	8	-4.333	74.368	21.198
44	CB	8	-4.463	75.782	18.223
45	N	9	-6.101	75.791	20.882
46	CA	9	-6.993	75.097	21.769

Table 16 continued

Atom Number	Atom type	Position in peptide	x	y	z
47	C	9	-8.036	74.312	20.948
48	O	9	-8.773	74.928	20.163
49	CB	9	-7.698	76.089	22.679
50	N	10	-8.021	72.999	21.144
51	CA	10	-8.966	72.137	20.488
52	C	10	-10.293	72.891	20.269
53	O	10	-10.367	73.727	19.356
54	CB	10	-9.213	70.899	21.332
55	N	11	-11.275	72.533	21.087
56	CA	11	-12.558	73.179	21.038
57	C	11	-13.561	72.288	20.278
58	O	11	-13.243	71.836	19.167
59	CB	11	-12.437	74.524	20.343
60	N	12	-14.696	72.054	20.925
61	CA	12	-15.750	71.281	20.326
62	C	12	0.000	-12.558	73.179
63	O	12	18.616	0.000	-12.558
64	CB	12	0.000	0.000	0.000

Table 17

Backbone 141					
Atom Number	Atom type	Position in peptide	x	y	z
0	N	0	0.000	0.000	0.000
1	CA	0	18.454	86.485	22.460
2	C	0	16.950	86.573	22.266
3	O	0	16.481	87.224	21.305
4	CB	0	0.000	0.000	0.000
5	N	1	16.227	85.893	23.151
6	CA	1	14.776	85.918	23.128
7	C	1	14.252	84.663	22.452
8	O	1	13.601	84.752	21.387
9	CB	1	14.299	87.132	22.349
10	N	2	14.573	83.520	23.055
11	CA	2	14.106	82.241	22.559
12	C	2	12.572	82.273	22.400
13	O	2	11.868	82.483	23.398
14	CB	2	14.499	81.135	23.523
15	N	3	12.141	82.099	21.156
16	CA	3	10.736	82.054	20.855
17	C	3	10.224	80.605	20.973
18	O	3	11.035	79.698	21.214
19	CB	3	10.489	82.573	19.449
20	N	4	8.911	80.468	20.833
21	CA	4	8.289	79.172	20.868
22	C	4	6.823	79.286	20.405
23	O	4	6.108	80.179	20.882
24	CB	4	8.338	78.611	22.279
25	N	5	6.465	78.404	19.478
26	CA	5	5.118	78.352	18.981
27	C	5	4.147	78.042	20.138
28	O	5	4.521	77.295	21.054
29	CB	5	4.999	77.280	17.911
30	N	6	2.972	78.656	20.055
31	CA	6	1.943	78.430	21.033
32	C	6	1.020	77.288	20.562
33	O	6	1.265	76.719	19.488
34	CB	6	1.130	79.697	21.234
35	N	7	0.034	76.991	21.401
36	CA	7	-0.938	75.983	21.081
37	C	7	-2.338	76.622	20.985
38	O	7	-2.577	77.649	21.637
39	CB	7	-0.939	74.903	22.150
40	N	8	-3.173	76.006	20.156
41	CA	8	-4.529	76.453	19.995
42	C	8	-5.492	75.437	20.641
43	O	8	-5.144	74.250	20.729
44	CB	8	-4.856	76.604	18.520
45	N	9	-6.629	75.957	21.087
46	CA	9	-7.649	75.129	21.670
47	C	9	-7.625	73.734	21.014

Table 17 continued

Atom Number	Atom type	Position in peptide	x	y	z
48	O	9	-6.531	73.205	20.765
49	CB	9	-9.013	75.766	21.470
50	N	10	-8.822	73.200	20.803
51	CA	10	-8.965	71.925	20.155
52	C	10	-10.460	71.616	19.939
53	O	10	-11.065	70.945	20.788
54	CB	10	-8.334	70.836	21.005
55	N	11	-10.983	72.148	18.840
56	CA	11	-12.353	71.910	18.476
57	C	11	-12.732	70.452	18.805
58	O	11	-12.400	69.551	18.020
59	CB	11	-12.548	72.168	16.992
60	N	12	-13.373	70.294	19.958
61	CA	12	-13.836	69.000	20.380
62	C	12	0.000	-12.353	71.910
63	O	12	18.541	0.000	-12.353
64	CB	12	0.000	0.000	0.000

Table 18

Backbone 144					
Atom Number	Atom type	Position in peptide	x	y	z
0	N	0	0.000	0.000	0.000
1	CA	0	18.480	86.428	22.392
2	C	0	16.967	86.551	22.343
3	O	0	16.431	87.361	21.553
4	CB	0	0.000	0.000	0.000
5	N	1	16.308	85.727	23.153
6	CA	1	14.861	85.759	23.256
7	C	1	14.262	84.643	22.416
8	O	1	13.512	84.919	21.454
9	CB	1	14.341	87.091	22.745
10	N	2	14.630	83.412	22.767
11	CA	2	14.106	82.241	22.093
12	C	2	12.565	82.287	22.092
13	O	2	11.968	82.501	23.158
14	CB	2	14.581	80.981	22.796
15	N	3	12.006	82.121	20.899
16	CA	3	10.578	82.090	20.743
17	C	3	10.094	80.628	20.667
18	O	3	10.880	79.754	20.273
19	CB	3	10.177	82.830	19.479
20	N	4	8.846	80.435	21.077
21	CA	4	8.236	79.135	21.020
22	C	4	6.879	79.228	20.292
23	O	4	6.338	80.337	20.167
24	CB	4	8.027	78.596	22.424
25	N	5	6.422	78.073	19.822
26	CA	5	5.148	77.990	19.162
27	C	5	4.052	77.645	20.190
28	O	5	4.068	76.532	20.737
29	CB	5	5.192	76.923	18.081
30	N	6	3.184	78.622	20.423
31	CA	6	2.076	78.436	21.319
32	C	6	1.134	77.348	20.765
33	O	6	1.402	76.819	19.676
34	CB	6	1.313	79.740	21.481
35	N	7	0.109	77.048	21.553
36	CA	7	-0.883	76.089	21.152
37	C	7	-2.256	76.780	21.027
38	O	7	-2.407	77.911	21.512
39	CB	7	-0.965	74.968	22.174
40	N	8	-3.167	76.084	20.357
41	CA	8	-4.509	76.574	20.198
42	C	8	-5.503	75.588	20.843
43	O	8	-5.193	74.391	20.931
44	CB	8	-4.832	76.735	18.722

Table 18 continued

Atom Number	Atom type	Position in peptide	x	y	z
45	N	9	-6.623	76.144	21.290
46	CA	9	-7.669	75.348	21.873
47	C	9	-8.201	74.343	20.832
48	O	9	-8.407	74.731	19.672
49	CB	9	-8.801	76.243	22.347
50	N	10	-8.360	73.106	21.286
51	CA	10	-8.894	72.067	20.448
52	C	10	-10.383	72.344	20.162
53	O	10	-11.124	72.681	21.097
54	CB	10	-8.745	70.719	21.133
55	N	11	-10.734	72.224	18.886
56	CA	11	-12.097	72.403	18.469
57	C	11	-12.907	71.126	18.774
58	O	11	-12.859	70.178	17.977
59	CB	11	-12.150	72.700	16.980
60	N	12	-13.575	71.155	19.921
61	CA	12	-14.414	70.059	20.322
62	C	12	0.000	-12.097	72.403
63	O	12	18.465	0.000	-12.097
64	CB	12	0.000	0.000	0.000

Example 4

The following method was used to identify high affinity binding peptides from Myelin Basic Protein (MBP). The binding affinities for a set of MBP peptides to HLA-DRB1*0401 have been experimentally determined and published. This set includes all possible 13 amino acid peptides from the MBP sequence which have a hydrophobic anchor residue at the P3 position. It is known that only such peptides bind to HLA-DR molecules with detectable affinity.

The same homology model of HLA-DRB1*0401 was used for this example as was used in Examples 1 and 2.

For each of the 13-mer peptides from the experimental determined set, a binding score was calculated as follows:

a) Calculate the steric overlap between the pocket bound peptide residue in the binding groove and an atom forming the pocket; this is value B.

b) Count the number of hydrogen bonds which could be formed between the pocket bound peptide residue and atoms forming the pocket; this is value C.

c) Calculate the strength of electrostatic interactions between any polar atoms of the pocket bound peptide residue and any polar atoms forming the pocket; this is value D.

d) Count the number of favourable contacts between the pocket bound peptide residue and atoms forming the pocket; this is value E.

e) These values were then transformed into a conformation score (Z) by using the following equation:

$$Z_n = cK_2C - cK_3D + cK_4E - cK_1B$$

Where K_1 to K_4 are constants and n is the sequence position of the peptide residue (numbered from 1 to the N-terminus to 13 at the C-terminus). K_1, K_2, K_3 and K_4 are equal to 100, 1500, 500 and 1000, respectively.

The conformation of each rotatable side-chain of the peptide residue was then altered by 15 degrees and the conformation score was recalculated.

The above steps were repeated for each residue of the peptide and the highest conformation score for each peptide residue was sued to determine the conformation score for the peptide.

At the point, the entire proceedings for establishing the conformation score for the peptide were repeated another 166 times, each time using a different peptide backbone form the library of peptide backbones.

The combination of peptide backbone and peptide side-chain conformations which gave the best conformation was then used to determine a binding score for the peptide.

The binding score was determined by establishing values of the following parameters:

a) Calculate the steric overlap between the pocket bound peptide residue in the binding groove and an atom forming the pocket; this is value B.

b) Count the number of hydrogen bonds which could be formed between the pocket bound peptide residue and atoms forming the pocket; this is value C.

c) Calculate the strength of electrostatic interactions between any polar atoms of the pocket bound peptide residue and any polar atoms forming the pocket; this is value D.

d) Count the number of favourable contacts between the pocket bound peptide residue and atoms forming the pocket; this is value E.

e) Calculate the hydrophobicity of the pocket bound peptide side chains using a hydrophobicity scale disclosed in Janin et al.

f) Calculate the number of MHC pocket residues which are paired with the pocket bound peptide residues. Pairing takes place if the centre of an atom from the MHC pocket residue and the centre of an atom from the pocket bound peptide residues are no more than the sum of their van der wall radii plus one Angstrom. The value A_n is calculated by summing the number of paired residues, where n is the number of the pocket. The values of A_n taking into account the pockets importance in binding are summed to give a value P.

The above values were then imported in to the following equation in order to determine the binding score (Y):

$$Y=P+bK_2C-bK_3D+bK_4E-bK_1B+bK_5He$$

Wherein the values bK_1, bK_2, bK_3, bK_4 and bK_5 are 2, 40, 600, 10 and 200 respectively.

As can be seen from the results in Table 19 the top four predicted scores pertain to four peptides which appear within the top five best binders.

Table 19

BB	PEPTIDE	AFFINITY	BINDING SCORE	D	E	F	B	P	Hc
104	HFFKNIVTPRTPP	40	4729	-0.12	11	17	97.7	3580	1.5
107	VHFFKNIVTPRTP	135	2125	-0.19	12	15	284.5	2255	0.2
104	PVVHFFKNIVTPR	161	4528	-0.06	15	12	337.6	4565	1.4
104	FSWGAEGQRPGFG	298	5205	-0.15	12	10	169.7	4670	-0.2
104	KGFKGVDAQGTLS	460	4353	-0.09	9	13	66.2	3145	1.9
112	KYLATASTMDHAR	479	2672	-0.09	13	15	106.8	1480	2.4
129	SKYLATASTMDHA	601	498	-0.06	11	13	275.7	620	0.4
141	RGLSLSRFSWGAE	1213	4140	-0.05	17	16	81.4	3455	1.7
62	TGILDSIGRFFGG	2942	337	0.04	21	17	25.3	-5	-0.6
0	RFFGGDRGAPKRG	3403	3218	-0.24	20	14	389.1	3100	1.6
104	NIVTPRTPPPSQG	6615	1971	0	10	11	305	2090	0.8
14	DSIGRFFGGDRGA	7268	1904	-0.08	8	15	37.3	1640	0.2
0	SRFSWGAEGQRPG	8352	1735	-0.08	20	13	466.8	1965	0.8
104	SKIFKLGGRDSRS	8494	1387	-0.1	10	10	149.2	825	2.8
118	SDYKSAHKGFKGV	8510	1864	-0.27	14	14	14.2	775	0.7
65	STMDHARHGFLPR	8860	1886	-0.21	14	15	191.3	1410	2.2
104	NPVVHFFKNIVTP	12870	1347	-0.11	12	10	332.5	1690	0.2
104	GTLSKIFKLGGRD	16000	4152	-0.11	17	10	118	3775	1.1
93	GRFFGGDRGAPKR	18467	244	-0.11	8	9	161	-175	2.3
75	KIFKLGGRDSRSG	25358	2185	-0.13	19	12	279.4	2060	1.4
0	FGYGGRASDYKSA	26397	1301	-0.12	15	15	306.1	1530	-0.4
0	PGFGYGGRASDYK	35200	3485	0.01	14	13	183.5	3165	1.4
144	GILDSIGRFFGGD	44400	2031	-0.09	21	14	32.1	1745	-0.5
134	KNIVTPRTPPPSQ	59000	1077	-0.04	9	10	45.9	340	3.1
0	KGVDAQGTLSKIF	100000	2067	-0.11	24	15	695.2	2795	0.3

KEY - BB = NUMBER OF THE BACKBONE CHOSEN FROM THE LIBRARY

CLAIMS

1. A method for the prediction of the binding affinity of a peptide to a major histocompatibility (MHC) class II molecules comprising;

a) ascertaining the characteristics of a MHC molecule binding groove,

b) presenting a selected peptide to the MHC molecule and ascertaining a first conformation score for each pocket bound peptide side-chain,

c) amending the conformation of each pocket bound peptide side-chain and ascertaining a second conformation score,

d) repeating step 3 with alternative conformations of each peptide pocket bound side-chain,

e) choosing the highest conformation score for each pocket bound peptide side-chain in each binding groove pockets, herein known as `the pocket', and

f) combining the highest conformation score for each pocket and ascertaining a binding score for the complete peptide.

2. A method according to claim 1 which further comprises the step of compiling information on all peptide fragments in a protein and comparing the binding scores.

3. A method according to any preceding claim wherein the conformation score is ascertained by at least one of the following parameters:

a) the number of favourable contacts between MHC residues forming one of the pockets and the pocket bound peptide residue; this is value E

b) the steric overlap between the pocket bound peptide residue bound in the pocket and an atom forming the pocket; this is value B,

c) the number of hydrogen bonds which could be formed between the pocket bound peptide residue and an atom forming the pocket; this is value C,

d) the strength of electrostatic interactions between any

polar atoms of the pocket bound peptide residue and any polar atoms forming the pocket; this is value D.

4. A method according to claim 3 wherein the steric overlap between the pocket bound peptide residue and the atoms forming the pocket can not be greater than 0.35 Angstroms.

5. A method according to claim 3 wherein a favourable contact occurs when an atom from an MHC residue and an atom from the peptide residue have their centres separated by no more than the sum of their radii plus 0.5 Angstroms and are not overlapping.

6. A method according to the preceding claims wherein values B to E are imported into a first equation, to give a conformation score (Z)

7. A method according to claim 6 wherein the first equation is $Z_n=(cK_2C)-(cK_3D)+(cK_4E)-(cK_1B)$, where cK_1 to cK_4 are constants and n is the number of the pocket.

8. A method according to claim 7 wherein cK_1 is between 50 and 150.

9. A method according to claim 7 wherein cK_2 is between 1000 and 2000.

10. A method according to claim 7 wherein cK_3 is between 250 and 750.

11. A method according to claim 7 wherein cK_4 is between 500 and 1500.

12. A method according to any preceding wherein the Z_n value for a pocket is multiplied by a coefficient, L, depending on the pockets importance in binding, to give a second Z_n value.

13. A method according to any of the preceding claims wherein all the Z values are summed to give a value J.

14. A method according to any of the preceding claims wherein the MHC residue is paired with the pocket-bound peptide residue if an atom from the MHC residue and an atom from the pocket-bound peptide residue have their centres separated by no more than the sum of their van der Waal radii plus one Angstrom.

15. A method according to claim 14 wherein a value A_n is calculated by summing the pairwise interaction frequencies of paired residues.

16. A method according to either claim 14 or 15 wherein the value A_n for a pocket is multiplied by a coefficient, X, depending on the pockets importance in binding.

17. A method according to claim 16 wherein the A_n value for the pockets are summed to give a value P.

18. A method according to any preceding claim wherein the binding score is ascertained by at least one of the following parameters

a) the number of groove-bound hydrophobic residues; this is value F,

b) the number of non groove-bound hydrophilic residues; this is value G,

c) the number of peptide residues deemed to fit within their respective binding pocket; this is value H.

19. A method according to any one of claims 13 to 18 wherein values F, G, H, J and P are imported into a second equation to give a first binding score, Y.

20. A method according to claim 19 wherein the second algorithm is $Y=J*F^2*(G*H+1)+ P$.

21. A method according to claim 1-17 wherein the hydrophobicity of the pocket bound peptide side chains is evaluated using a hydrophobicity scale; this is value He.

22. A method according to claim 21 wherein the hydrophobicity scale ranges from -1.8 for lysine to 0.9 for cysteine.

23. A method according to either of claims 21 or 22 wherein $Y=(bK_2C)-(bK_3\ D)+(bK_4E)-(bK_1B)+(bK_5He)+P$.

24. A method according to claim 23 wherein bK_1 is between 1 and 5.

25. A method according to claim 23 wherein bK_2 is between 20 and 60.

26. A method according to claim 23 wherein bK_3 is between 300 and 900.

27. A method according to claim 23 wherein bK_4 is between 1 and 20.

28. A method according to claim 23 wherein bK_5 is between 1 and 800.

29. A method according to any preceding claim wherein the steps in claim 3 are repeated for each pocket and each conformation of the peptide residue in said pocket.

30. A method according to claim 29 wherein the conformation of the peptide is altered by rotating a side chain of the peptide residue by a pre-determined amount.

31. A method according to either claim 29 or 30 where in the conformation of the peptide is altered by changing the conformation of the peptide backbone.

32. A method according to any preceding claim wherein the steps are repeated using different peptides from a protein.

33. A method according to any of the preceding claim wherein the binding scores (Y) for different peptides are tabulated and compared.

34. A method according to any of the preceding claim which is used in the manufacture of a vaccine derived from a peptide identified by said method.

35. A method according to any of the preceding claims which is used to remove potentially immunogenic sequences from a protein and thus reduce said proteins immunogenicity when administered to an organism.

36. A computer conditioned to receive information characterising a peptide bound to the MHC molecule and to utilise said information to perform a procedure having the following steps;

a) ascertaining the characteristics of a MHC molecule binding groove;

b) presenting a selected peptide, which is selected by a predetermined program, to the MHC molecule and ascertaining a first conformation score;

c) amending the conformation of the peptide, by way of a predetermined program, and ascertaining a second conformation score;

d) repeating step 3 with other conformations of the peptide;

e) selecting the peptide conformation with the highest conformation score; and

f) calculating the binding score from the conformation score.

37. A computer according to claim 36 further comprising a step (7) which comprises repeating steps 1-4 with other peptide fragments in the protein

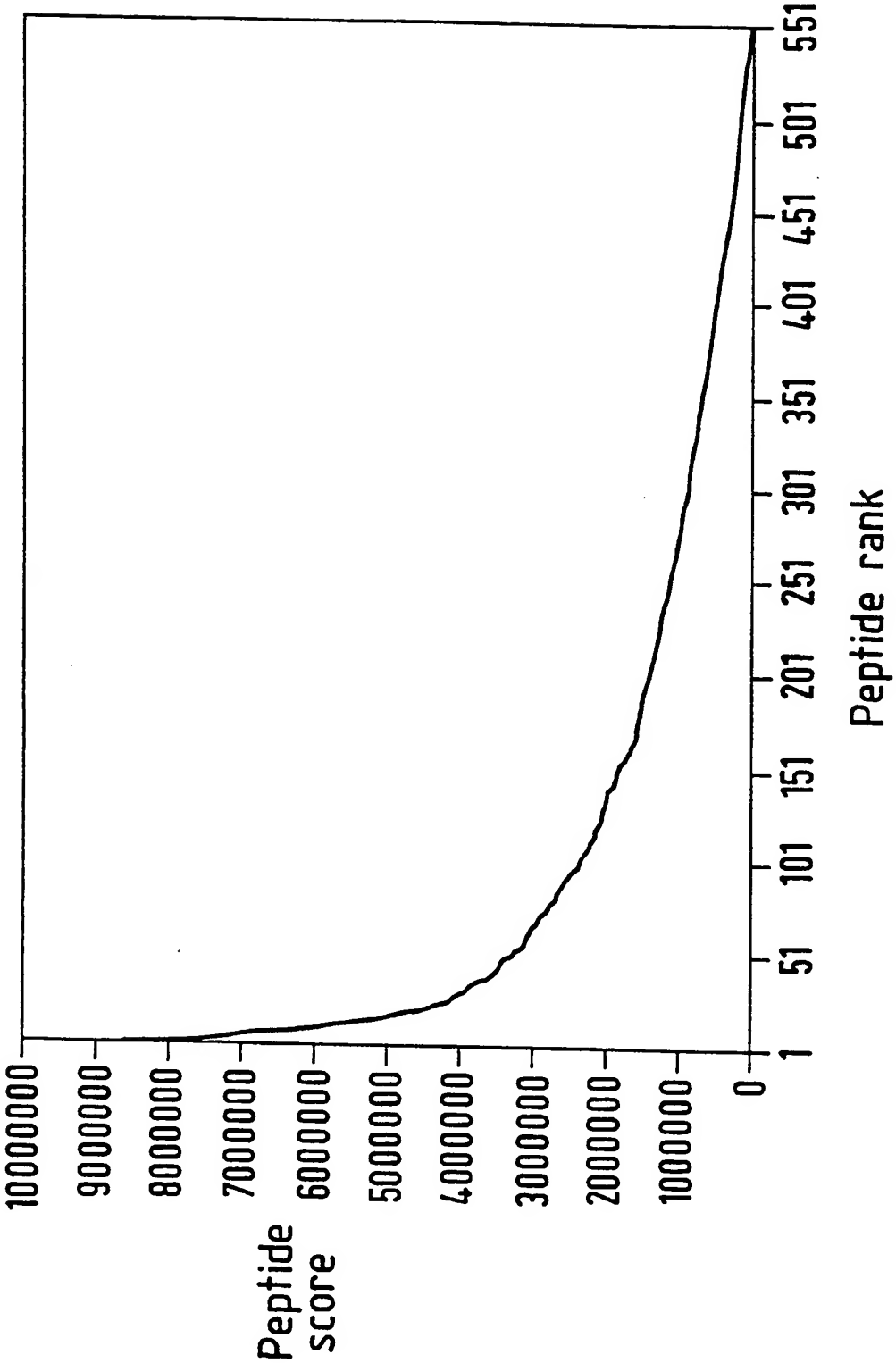
to generate information on all peptide fragments in a protein

so that a comparison can be made of the strength of the binding between the peptide and the MHC molecule.

38. A computer according to either claim 36 or 37 further comprising a step (8) which comprises altering the conformation of the backbone of the peptide fragment.

39. A pharmaceutical composition produced resultant upon to a method as claimed in anyone of claims 1 to 35.

FIG. 1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8000000
7000000
6000000
5000000
4000000
3000000
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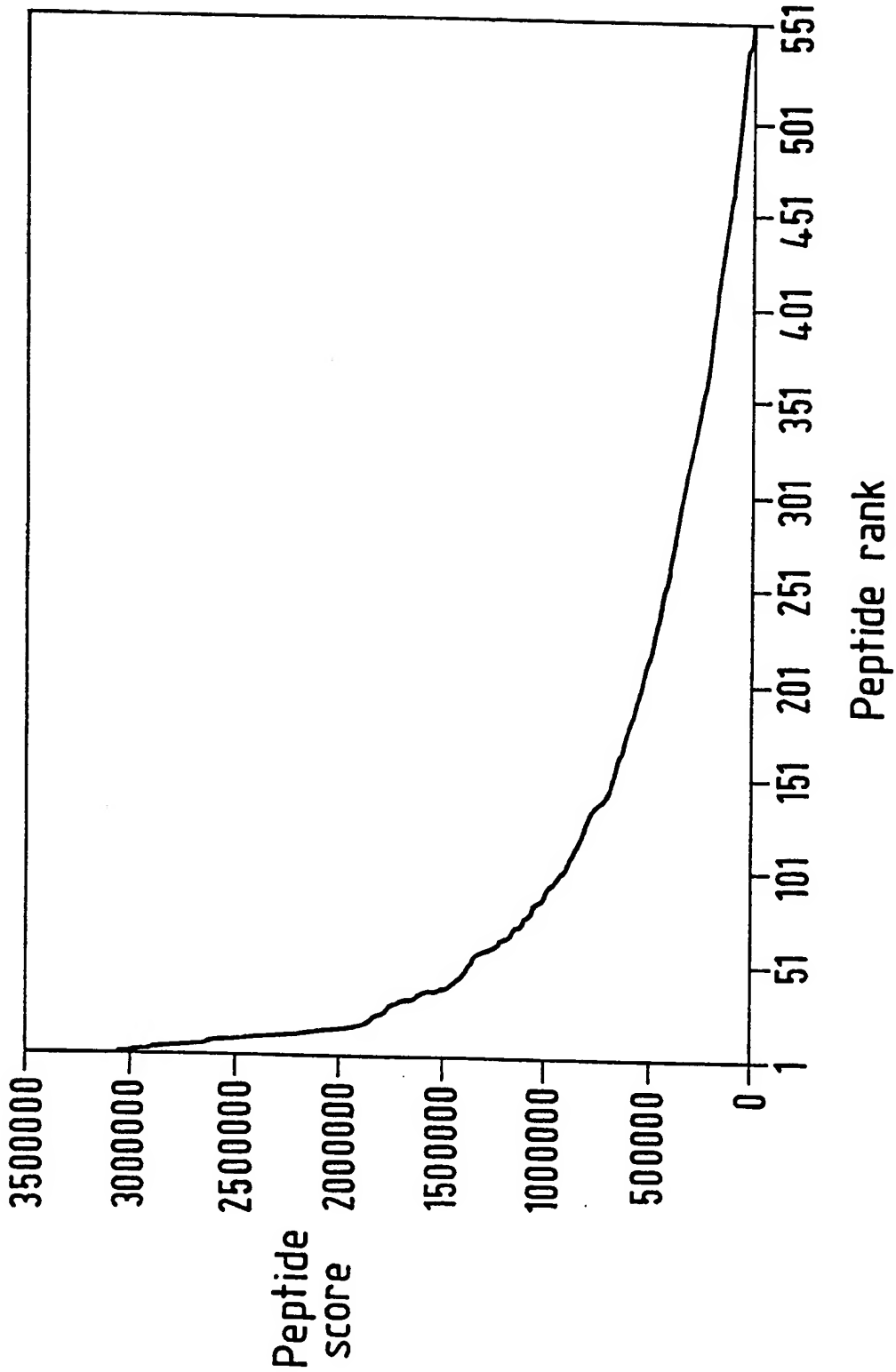
0
1
51
101
151
201
251
301
351
401
451
501
551
Peptide rank

FIG. 2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3000000
2500000
2000000
1500000
1000000
500000
0
Peptide score
1
51
101
151
201
251
301
351
401
451
501
551
Peptide rank

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/01801

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/569 G01N33/564 G01N33/566 C07K14/705

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 31483 A (ECLAGEN LTD) 23 November 1995 see page 2, line 23 - line 28 see page 5, line 5 - line 12	1-35
X	---	39
X,P	WO 97 40852 A (ANERGEN INC) 6 November 1997 see claims 31,32	39
A,P	---	1-35
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 October 1998	05/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van Bohemen, C

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/01801

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	T.E. JOHANSEN ET AL.: "Peptide binding to MHC class I is determined by individual pockets in the binding groove." SCANDINAVIAN JOURNAL OF IMMUNOLOGY, vol. 46, no. 2, 1 August 1997, pages 137-146, XP002081826 oxford uk see the whole document	1-35,39

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/01801

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 36-38
because they relate to subject matter not required to be searched by this Authority, namely:
Rule 39.1(i) PCT - Mathematical method

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

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INTERNATIONAL SEARCH REPORT

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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(57) Abstract

The present invention relates to a novel improved protein mutant which produces low allergenic response in humans compared to the parent of that mutant. Specifically, the present invention comprises neutralizing or reducing the ability of T–cells to recognize epitopes and thus prevent sensitization of an individual to the protein.

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MUTANT PROTEINS HAVING LOWER ALLERGENIC RESPONSE IN HUMANS AND METHODS FOR CONSTRUCTING, IDENTIFYING AND PRODUCING SUCH PROTEINS

BACKGROUND OF THE INVENTION

A. Field of the Invention

The present invention relates to proteins which produce lower allergenic response in humans exposed to such proteins, and an assay predictive of such response. More specifically, the present invention relates to a novel improved protein mutant which produces very low allergenic response in humans sensitized to that protein through exposure compared to the precursor of such protein mutant.

B. State of the Art

Proteins used in industrial, pharmaceutical and commercial applications are of increasing prevalence. As a result, the increased exposure due to this prevalence has been responsible for some safety hazards caused by the sensitization of certain persons to those peptides, whereupon subsequent exposure causes extreme allergic reactions which can be injurious and even fatal. For example, proteases are known to cause dangerous hypersensitivity in some individuals. As a result, despite the usefulness of proteases in industry, e.g., in laundry detergents, cosmetics, textile treatment etc...., and the extensive research performed in the field to provide improved proteases which have, for example, more effective stain removal under detergency conditions, the use of proteases in industry has been problematic due to their ability to produce a hypersensitive allergenic response in some humans.

Much work has been done to alleviate these problems. Among the strategies explored to reduce immunogenic potential of protease use have been improved production processes which reduce potential contact by controlling and minimizing workplace concentrations of dust particles or aerosol carrying airborne protease, improved granulation processes which reduce the amount of dust or aerosol actually produced from the protease product, and improved recovery processes to reduce the level of potentially allergenic contaminants in the final product. However, efforts to reduce the allergenicity of protease, per se, have been relatively unsuccessful. Alternatively, efforts have been made to mask epitopes in protease which are recognized by immunoglobulin E (IgE) in hypersensitive individuals (PCT Publication No. WO 92/10755) or to enlarge or change the nature of the antigenic determinants by attaching polymers or peptides/proteins to the problematic protease.

When an adaptive immune response occurs in an exaggerated or inappropriate form, the individual experiencing the reaction is said to be hypersensitive. Hypersensitivity reactions are the result of normally beneficial immune responses acting inappropriately and sometimes cause inflammatory reactions and tissue damage. They can be provoked by many antigens; and the cause of a hypersensitivity reaction will vary from one individual to the next. Hypersensitivity does not normally manifest itself upon first contact with the antigen, but usually appears upon subsequent contact. One form of hypersensitivity occurs when an IgE response is directed against innocuous environmental antigens, such as pollen, dust-mites or animal dander. The resulting release of pharmacological mediators by IgE-sensitized mast cells produces an acute inflammatory reaction with symptoms such as asthma or rhinitis.

Nonetheless, a strategy comprising modifying the IgE sites will not generally be successful in preventing the cause of the initial sensitization reaction. Accordingly, such strategies, while perhaps neutralizing or reducing the severity of the subsequent hypersensitivity reaction, will not reduce the number or persons actually sensitized. For example, when a person is known to be hypersensitive to a certain antigen, the general, and only safe, manner of dealing with such a situation is to isolate the hypersensitive person from the antigen as completely as possible. Indeed, any other course of action would be dangerous to the health of the hypersensitive individual. Thus, while reducing the danger of a specific protein for a hypersensitive individual is important, for industrial purposes it would be far more valuable to render a protein incapable of initiating the hypersensitivity reaction in the first place.

T-lymphocytes (T-cells) are key players in the induction and regulation of immune responses and in the execution of immunological effector functions. Specific immunity against infectious agents and tumors is known to be dependent on these cells and they are believed to contribute to the healing of injuries. On the other hand, failure to control these responses can lead to auto aggression. In general, antigen is presented to T-cells in the form of antigen presenting cells which, through a variety of cell surface mechanisms, capture and display antigen or partial antigen in a manner suitable for antigen recognition by the T-cell. Upon recognition of a specific epitope by the receptors on the surface of the T-cells (T-cell receptors), the T-cells begin a series of complex interactions, including proliferation, which result in the production of antibody by B-cells. While T-cells and B-cells are both activated by antigenic epitopes which exist on a given protein or peptide, the actual epitopes recognized by these mononuclear cells are generally not identical. In fact, the epitope which activates a T-cell to initiate the creation

of immunologic diversity is quite often not the same epitope which is later recognized by B-cells in the course of the immunologic response. Thus, with respect to hypersensitivity, while the specific antigenic interaction between the T-cell and the antigen is a critical element in the initiation of the immune response to antigenic exposure, the specifics of that interaction, i.e., the epitope recognized, is often not relevant to subsequent development of a full blown allergic reaction.

PCT Publication No. WO 96/40791 discloses a process for producing polyalkylene oxide-polypeptide conjugates with reduced allergenicity using polyalkylene oxide as a starting material.

reaction has been initiated, sensitization has already occurred. Accordingly, there is a need for a method of determining epitopes which cause sensitization in the first place, as neutralization of these epitopes will result in significantly less possibility for sensitization to occur, thus reducing the possibility of initial sensitization.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a protein having decreased potential to cause allergenic response in humans compared to a precursor protein.

It is a further object of the present invention to provide for a protease variant which has useful activity in common protease applications, such as detergents and or the treatment of wool to prevent felting, in bar or liquid soap applications, dish-care formulations, contact lens cleaning solutions or products, peptide hydrolysis, waste treatment, textile applications such as anti-felting, in cosmetic formulations and for skin care, or as fusion-cleavage enzymes in protein production, which protease variant can be more safely used due to its lowered allergenic potential.

According to the present invention, a method for identifying T-cell epitopes within a protein is provided. The present invention provides an assay which identifies epitopes as follows: antigen presenting cells are combined with naïve human T-cells and with a peptide of interest. In a preferred embodiment of the invention, a method is provided wherein a T-cell epitope is recognized comprising the steps of: (a) obtaining from a single blood source a solution of dendritic cells and a solution of naïve CD4+ and/or CD8+ T-cells; (b) promoting differentiation in said solution of dendritic cells; (c) combining said solution of differentiated dendritic cells and said naïve CD4+ and/or CD8+ T-cells with a peptide of interest; (d) measuring the proliferation of T-cells in said step (c).

According to another embodiment of the present invention, a protein is provided in which a T-cell epitope is modified so as to reduce or preferably neutralize (eliminate) the ability of the T-cell to identify that epitope. Thus, a protein is provided having reduced allergenicity, wherein said protein comprises a modification comprising the substitution or deletion of amino acid residues which are identified as within a T-cell epitope. According to a preferred embodiment, an epitope is determined in a protein or peptide which, when recognized by a T-cell, results in the proliferation of T-cells which is greater than the baseline. That T-cell epitope is then modified so that, when the peptide comprising the epitope is analyzed in the assay of the invention, it results in lesser proliferation than the protein comprising the unmodified epitope. More preferably, the epitope to be modified produces greater than three times the baseline T-cell proliferation in a sample. When

modified, the epitope produces less than three times the baseline T-cell proliferation, preferably less than two times the baseline T-cell proliferation and most preferably less than or substantially equal to the baseline T-cell proliferation in a sample.

Preferably, the epitope is modified in one of the following ways: (a) the amino acid sequence of the epitope is substituted with an analogous sequence from a human homolog to the protein of interest, i.e., human subtilisin or another human protease derived subtilisin like molecule such as furin or the kexins (see e.g., *Methods in Enzymology*, Vol. 244., (1994) pp. 175 *et seq*; Roebroek et al., *EMBO J.*, Vol. 5, No. 9, pp. 2197-2202 (1986); Tomkinson et al., *Biochem.*, Vol. 30, pp. 168-174 (1991); Keifer et al., *DNA and Cell Biol.*, Vol. 10, No. 10, pp. 757-769 (1991)); (b) the amino acid sequence of the epitope is substituted with an analogous sequence from a non-human homolog to the protein of interest, which analogous sequence produces a lesser allergenic response due to T-cell recognition than that of the protein of interest; (c) the amino acid sequence of the epitope is substituted with a sequence which substantially mimics the major tertiary structure attributes of the epitope, but which produces a lesser allergenic response due to T-cell recognition than that of the protein of interest; or (d) with any sequence which produces lesser allergenic response due to T-cell recognition than that of the protein of interest.

In a specific embodiment of the invention, a protease variant is provided comprising at least one amino acid substitution at a position corresponding to residues 170, 171, 172 and/or 173 in BPN', wherein such substitutions comprise modifying residue 170 to aspartic acid, modifying residue 171 to glutamine, modifying residue 172 to methionine and/or modifying residue 173 to aspartic acid. In a most preferred embodiment, the substitution comprises modifying residues 170, 171 and 173 to aspartic acid, glutamine and aspartic acid, respectively.

In another embodiment of the present invention, a method for producing the protein of the invention having reduced allergenicity is provided. Preferably, the mutant protein is prepared by modifying a DNA encoding a precursor protein so that the modified DNA encodes the mutant protein of the invention.

In yet another embodiment of the invention, DNA sequences encoding the mutant protein, as well as expression vectors containing such DNA sequences and host cells transformed with such vectors are provided, which host cells are preferably capable of expressing such DNA to produce the mutant protein of the invention either intracellularly or extracellularly.

The mutant protein of the invention is useful in any composition or process in which the precursor protein is generally known to be useful. For example, where the protein is a protease, the reduced allergenicity protease can be used as a component in cleaning products such as laundry detergents and hard surface cleansers, as an aid in the preparation of leather, in the treatment of textiles such as wool and/or silk to reduce felting, as a component in a personal care, cosmetic or face cream product, and as a component in animal or pet feed to improve the nutritional value of the feed. Similarly, where the protein is an amylase, the reduce allergenicity amylase can be used for the liquefaction of starch, as a component in a dishwashing detergent, for desizing of textiles, in a laundry detergent or any other use for which amylase is useful.

One advantage of the present invention is that by measuring the proliferation of T-cells due to T-cell epitope recognition, it is possible to identify peptides which contain epitopes responsible for initially sensitizing an individual. That is, T-cell proliferation due to T-cell epitope recognition results in sensitization of an individual to that peptide or a protein which contains it. Neutralization of such "sensitizing" T-cell epitopes will inevitably result in a greater degree of safety for those who handle or are otherwise exposed to the antigen containing the epitope because they will not be initially sensitized, thus preventing the production of Ig antibodies typical of an allergic reaction upon subsequent exposure to the antigen.

An advantage of the present invention is the preparation of proteins, including enzymes, which may be used with significantly less danger of sensitization for the individuals exposed. Thus, for example, the proteins of the invention may be more safely used in cosmetics such as face creams, detergents such as laundry detergents, hard surface cleaning compositions and pre-wash compositions or any other use of protein, including enzymes, wherein human exposure is a necessary by-product.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1 A, B1, B2 and B3 illustrate the DNA (SEQ ID:NO 1) and amino acid (SEQ ID: NO 2) sequence for *Bacillus amyloliquefaciens* subtilisin (BPN') and a partial restriction map of this gene.

Fig. 2 illustrates the conserved amino acid residues among subtilisins from *Bacillus amyloliquefaciens* (SEQ ID:NO 3) and *Bacillus lentus* (wild-type) (SEQ ID:NO 4).

Figs. 3A and 3B illustrate an amino acid sequence alignment of subtilisin type proteases from *Bacillus amyloliquefaciens* (BPN'), *Bacillus subtilis*, *Bacillus licheniformis*

(SEQ ID:NO 5) and *Bacillus lentus*. The symbol * denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

Fig. 4 illustrates the additive T-cell response of 16 peripheral mononuclear blood samples to peptides corresponding to the *Bacillus lentus* protease. Peptide E05 includes the region comprising residues corresponding to 170-173 in protease from *Bacillus amyloliquefaciens*.

Fig. 5 illustrates the additive T-cell response of 10 peripheral mononuclear blood samples to peptides corresponding to the human subtilisin molecule. Peptides F10, F9, F8 and F7 all contain the amino acid sequence DQMD corresponding to the region comprising residues corresponding to 170-173 in protease from *Bacillus amyloliquefaciens* in the sequence alignment of Fig. 3.

Fig. 6A and 6B/6C illustrate amino acid strings corresponding to peptides derived from the sequence of *Bacillus lentus* protease and a human subtilisin, respectively.

Fig. 7 illustrates the amino acid sequence of human subtilisin (SEQ ID:NO 6).

Fig. 8 illustrates an amino acid sequence alignment of BPN' (*Bacillus amyloliquefaciens*) protease, SAVINASE (*Bacillus lentus*) protease and human subtilisin (S2HSBT).

Fig. 9 illustrates the T-cell response to peptides derived from *Bacillus lentus* protease in a sample taken from an individual known to be hypersensitive to *Bacillus lentus* protease. Peptide E05 represents the region corresponding to 170-173 in protease from *Bacillus amyloliquefaciens.*

Fig. 10 illustrates the T-cell response to various alanine substitutions in the E05 *Bacillus lentus* protease peptide set in a sample taken from an individual known to be hypersensitive to *Bacillus lentus* protease.

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, a method for identifying T-cell epitopes is provided. The present invention provides an assay which identifies epitopes as follows: differentiated dendritic cells are combined with naïve human CD4+ and/or CD8+ T-cells and with a peptide of interest. More specifically, a method is provided wherein a T-cell epitope is recognized comprising the steps of: (a) obtaining from a single blood source a solution of dendritic cells and a solution of naïve CD4+ and/or CD8+ T-cells; (b) promoting differentiation in said solution of dendritic cells; (c) combining said solution of differentiated

dendritic cells and said naïve CD4+ and/or CD8+ T-cells with a peptide of interest; (d) measuring the proliferation of T-cells in said step (c).

The peptide of interest to be analyzed according to the assay of the invention is derived from a protein or enzyme for which reduced allergenicity is desirable or required. In the practice of the invention, it is possible to identify with precision the location of an epitope which can cause sensitization in an individual or sampling of individuals. In a particularly effective embodiment of the invention, a series of peptide oligomers which correspond to all or part of the protein or enzyme are prepared. For example, a peptide library is produced covering the relevant portion or all of the protein. One particularly useful manner of producing the peptides is to introduce overlap into the peptide library, for example, producing a first peptide corresponds to amino acid sequence 1-10 of the subject protein, a second peptide corresponds to amino acid sequence 4-14 of the subject protein, a third peptide corresponds to amino acid sequence 7-17 of the subject protein, a fourth peptide corresponds to amino acid sequence 10-20 of the subject protein etc....until representative peptides corresponding to the entire molecule are created. By analyzing each of the peptides individually in the assay provided herein, it is possible to precisely identify the location of epitopes recognized by T-cells. In the example above, the reaction of one specific peptide to a greater extent than it's neighbors will facilitate identification of the epitope anchor region to within three amino acids. After determining the location of these epitopes, it is possible to alter the amino acids within each epitope until the peptide produces a less significant T-cell response.

"Antigen presenting cell" as used herein means a cell of the immune system which present antigen on their surface which is recognizable by receptors on the surface of T-cells. Examples of antigen presenting cells are dendritic cells, interdigitating cells, activated B-cells and macrophages.

"T-cell proliferation" as used herein means the number of T-cells produced during the incubation of T-cells with the antigen presenting cells, with or without antigen.

"Baseline T-cell proliferation" as used herein means T-cell proliferation which is normally seen in an individual in response to exposure to antigen presenting cells in the absence of peptide or protein antigen. For the purposes herein, the baseline T-cell proliferation level was determined on a per sample basis for each individual as the proliferation of T-cells in response to antigen presenting cells in the absence of antigen.

"T-cell epitope" means a feature of a peptide or protein which is recognized by a T-cell receptor in the initiation of an immunologic response to the peptide comprising that antigen. Recognition of a T-cell epitope by a T-cell is generally believed to be via a

mechanism wherein T-cells recognize peptide fragments of antigens which are bound to class I or class II major histocompatability (MHC) molecules expressed on antigen-presenting cells (see e.g., Moeller, G. ed., Antigenic Requirements for Activation of MHC-Restricted Responses, *Immunological Review*, Vol. 98, p. 187 (Copenhagen; Munksgaard) (1987).

The epitopes determined according to the assay provided herein are then modified to reduce the allergenic potential of the protein of interest. In a preferred embodiment, the epitope to be modified produces a level of T-cell proliferation of greater than three times the baseline T-cell proliferation in a sample. When modified, the epitope produces less than three times the baseline proliferation, preferably less than two times the baseline proliferation and most preferably less than or substantially equal to the baseline proliferation in a sample.

Preferably, the epitope is modified in one of the following ways: (a) the amino acid sequence of the epitope is substituted with an analogous sequence from a human homolog to the protein of interest; (b) the amino acid sequence of the epitope is substituted with an analogous sequence from a non-human homolog to the protein of interest, which analogous sequence produces a lesser allergenic response due to T-cell epitope recognition than that of the protein of interest; (c) the amino acid sequence of the epitope is substituted with a sequence which substantially mimics the major tertiary structure attributes of the epitope, but which produces a lesser allergenic response due to T-cell epitope recognition than that of the protein of interest; or (d) with any sequence which produces lesser allergenic response due to T-cell epitope recognition than that of the protein of interest.

"Sample" as used herein comprises mononuclear cells which are naïve, i.e., not sensitized, to the antigen in question.

"Homolog" as used herein means a protein or enzyme which has similar catalytic action, structure and/or use as the protein of interest. It is desirable to find a homolog that has a tertiary and/or primary structure similar to the protein of interest as replacement of the epitope in the protein of interest with an analogous segment from the homolog will reduce the disruptiveness of the change. Thus, closely homologous enzymes will provide the most desirable source of epitope substitutions. Alternatively, if possible, it is advantageous to look to human analogs for a given protein. For example, substituting a specific epitope in a bacterial subtilisin with a sequence from a human analog to subtilisin (i.e., human subtilisin) should result in less allergenicity in the bacterial protein.

An "analogous" sequence may be determined by ensuring that the replacement amino acids show a similar function, the tertiary structure and/or conserved residues to the amino acids in the protein of interest at or near the epitope. Thus, where the epitope region contains, for example, an alpha-helix or a beta-sheet structure, the replacement amino acids should maintain that specific structure.

While the present invention extends to all proteins for which it is desired to reduce allergenicity, for the sake of simplicity, the following will describe a particularly preferred embodiment of the invention, the modification of protease. Proteases are carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "protease" means a naturally-occurring protease or a recombinant protease. Naturally-occurring proteases include α-aminoacylpeptide hydrolase, peptidylamino acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallocarboxypeptidase, thiol proteinase, carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exo-proteases.

Subtilisins are bacterial or fungal proteases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally-occurring subtilisin or a recombinant subtilisin. A series of naturally-occurring subtilisins is known to be produced and often secreted by various microbial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus, is aspartate-histidine-serine. In the chymotrypsin related proteases, the relative order, however, is histidine-aspartate-serine. Thus, subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases. Examples include but are not limited to the subtilisins identified in Fig. 3 herein. Generally and for purposes of the present invention, numbering of the amino acids in proteases corresponds to the numbers assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1.

"Recombinant subtilisin" or "recombinant protease" refer to a subtilisin or protease in which the DNA sequence encoding the subtilisin or protease is modified to produce a variant (or mutant) DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally-occurring amino acid sequence. Suitable

methods to produce such modification, and which may be combined with those disclosed herein, include those disclosed in US Patent 4,760,025 (RE 34,606), US Patent 5,204,015 and US Patent 5,185,258.

"Non-human subtilisins" and the DNA encoding them may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram negative organisms such as *E. coli* or *Pseudomonas* and gram positive bacteria such as *Micrococcus* or *Bacillus*. Examples of eucaryotic organisms from which subtilisin and their genes may be obtained include yeast such as *Saccharomyces cerevisiae*, fungi such as *Aspergillus* sp.

"Human subtilisin" means proteins of human origin which have subtilisin type catalytic activity, e.g., the kexin family of human derived proteases. An example of such a protein is represented by the sequence in Fig. 7. Additionally, derivatives or homologs of human subtilisin, including those from non-human sources such as mouse or rabbit, which retain the essential ability to hydrolyze peptide bonds and have at least 50%, preferably at least 65% and most preferably at least 80% homology to the protein of Fig. 7 are considered human subtilisins for the purpose of the invention.

A "protease variant" has an amino acid sequence which is derived from the amino acid sequence of a "precursor protease". The precursor proteases include naturally-occurring proteases and recombinant proteases. The amino acid sequence of the protease variant is "derived" from the precursor protease amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor protease rather than manipulation of the precursor protease enzyme *per se*. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein, as well as methods known to those skilled in the art (see, for example, EP 0 328299, WO89/06279 and the US patents and applications already referenced herein).

The amino acid position numbers used herein refer to those assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1. The invention, however, is not limited to the mutation of this particular subtilisin but extends to precursor proteases containing amino acid residues at positions which are "equivalent" to the particular identified residues in *Bacillus amyloliquefaciens* subtilisin. In a preferred embodiment of the present invention, the precursor protease is *Bacillus lentus* subtilisin and the substitutions, deletions or insertions are made at the equivalent amino acid residue in *B. lentus* corresponding to those listed above.

A residue (amino acid) of a precursor protease is equivalent to a residue of *Bacillus amyloliquefaciens* subtilisin if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or analogous to a specific residue or portion of that residue in *Bacillus amyloliquefaciens* subtilisin (i.e., having the same or similar functional capacity to combine, react, or interact chemically).

In order to establish homology to primary structure, the amino acid sequence of a precursor protease is directly compared to the *Bacillus amyloliquefaciens* subtilisin primary sequence and particularly to a set of residues known to be invariant in subtilisins for which the sequence is known. For example, Fig. 2 herein shows the conserved residues as between *B. amyloliquefaciens* subtilisin and *B. lentus* subtilisin. After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of *Bacillus amyloliquefaciens* subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained.

For example, the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus licheniformis* (*carlsbergensis*) and *Bacillus lentus* can be aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in each sequence. The conserved residues as between BPN' and *B. lentus* are identified in Fig. 2.

These conserved residues, thus, may be used to define the corresponding equivalent amino acid residues of *Bacillus amyloliquefaciens* subtilisin in other subtilisins such as subtilisin from *Bacillus lentus* (PCT Publication No. W089/06279 published July 13, 1989), the preferred protease precursor enzyme herein, or the subtilisin referred to as PB92 (EP 0 328 299), which is highly homologous to the preferred *Bacillus lentus* subtilisin. The amino acid sequences of certain of these subtilisins are aligned in Figs. 3A and 3B with the sequence of *Bacillus amyloliquefaciens* subtilisin to produce the maximum homology of conserved residues. As can be seen, there are a number of deletions in the sequence of *Bacillus lentus* as compared to *Bacillus amyloliquefaciens* subtilisin. Thus, for example, the equivalent amino acid for Val165 in *Bacillus amyloliquefaciens* subtilisin in the other subtilisins is isoleucine for *B. lentus* and *B. licheniformis*.

Thus, for example, the amino acid at position +170 is lysine (K) in both *B. amyloliquefaciens* and *B. licheniformis* subtilisins and arginine (R) in Savinase. In one embodiment of the protease variants of the invention, however, the amino acid equivalent to +170 in *Bacillus amyloliquefaciens* subtilisin is substituted with aspartic acid (D). The abbreviations and one letter codes for all amino acids in the present invention conform to the PatentIn User Manual (GenBank, Mountain View, CA) 1990, p.101.

"Equivalent residues" may also be defined by determining homology at the level of tertiary structure for a precursor protease whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the precursor protease and *Bacillus amyloliquefaciens* subtilisin (N on N, CA on CA, C on C and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the protease in question to the *Bacillus amyloliquefaciens* subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

$$R\,factor = \frac{\Sigma_h |Fo(h)| - |Fc(h)|}{\Sigma_h |Fo(h)|}$$

Equivalent residues which are functionally analogous to a specific residue of *Bacillus amyloliquefaciens* subtilisin are defined as those amino acids of the precursor protease which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the *Bacillus amyloliquefaciens* subtilisin. Further, they are those residues of the precursor protease (for which a tertiary structure has been obtained by x-ray crystallography) which occupy an analogous position to the extent that, although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie with 0.13nm of the corresponding side chain atoms of *Bacillus amyloliquefaciens* subtilisin. The coordinates of the three dimensional structure of *Bacillus amyloliquefaciens* subtilisin are set forth in EPO Publication No. 0 251 446 (equivalent to US Patent 5,182,204, the disclosure of which is incorporated herein by

reference) and can be used as outlined above to determine equivalent residues on the level of tertiary structure.

Some of the residues identified for substitution, insertion or deletion are conserved residues whereas others are not. In the case of residues which are not conserved, the replacement of one or more amino acids is limited to substitutions which produce a variant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such replacements should not result in a naturally-occurring sequence. The protease variants of the present invention include the mature forms of protease variants, as well as the pro- and prepro- forms of such protease variants. The prepro- forms are the preferred construction since this facilitates the expression, secretion and maturation of the protease variants.

"Prosequence" refers to a sequence of amino acids bound to the N-terminal portion of the mature form of a protease which when removed results in the appearance of the "mature" form of the protease. Many proteolytic enzymes are found in nature as translational proenzyme products and, in the absence of post-translational processing, are expressed in this fashion. A preferred prosequence for producing protease variants is the putative prosequence of *Bacillus amyloliquefaciens* subtilisin, although other protease prosequences may be used.

A "signal sequence" or "presequence" refers to any sequence of amino acids bound to the N-terminal portion of a protease or to the N-terminal portion of a proprotease which may participate in the secretion of the mature or pro forms of the protease. This definition of signal sequence is a functional one, meant to include all those amino acid sequences encoded by the N-terminal portion of the protease gene which participate in the effectuation of the secretion of protease under native conditions. The present invention utilizes such sequences to effect the secretion of the protease variants as defined herein. One possible signal sequence comprises the first seven amino acid residues of the signal sequence from *Bacillus subtilis* subtilisin fused to the remainder of the signal sequence of the subtilisin from *Bacillus lentus* (ATCC 21536).

A "prepro" form of a protease variant consists of the mature form of the protease having a prosequence operably linked to the amino terminus of the protease and a "pre" or "signal" sequence operably linked to the amino terminus of the prosequence.

"Expression vector" refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable

mRNA ribosome binding sites and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

The "host cells" used in the present invention generally are procaryotic or eucaryotic hosts which preferably have been manipulated by the methods disclosed in US Patent 4,760,025 (RE 34,606) to render them incapable of secreting enzymatically active endoprotease. A preferred host cell for expressing protease is the *Bacillus* strain BG2036 which is deficient in enzymatically active neutral protease and alkaline protease (subtilisin). The construction of strain BG2036 is described in detail in US Patent 5,264,366. Other host cells for expressing protease include *Bacillus subtilis* I168 (also described in US Patent 4,760,025 (RE 34,606) and US Patent 5,264,366, the disclosure of which are incorporated herein by reference), as well as any suitable *Bacillus* strain such as *B. licheniformis, B. lentus*, etc.

Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of either replicating vectors encoding the protease variants or expressing the desired protease variant. In the case of vectors which encode the pre- or prepro-form of the protease variant, such variants, when expressed, are typically secreted from the host cell into the host cell medium.

"Operably linked", when describing the relationship between two DNA regions, simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The genes encoding the naturally-occurring precursor protease may be obtained in accord with the general methods known to those skilled in the art. The methods generally comprise synthesizing labeled probes having putative sequences encoding regions of the protease of interest, preparing genomic libraries from organisms expressing the protease,

and screening the libraries for the gene of interest by hybridization to the probes. Positively hybridizing clones are then mapped and sequenced.

The cloned protease is then used to transform a host cell in order to express the protease. The protease gene is then ligated into a high copy number plasmid. This plasmid replicates in hosts in the sense that it contains the well-known elements necessary for plasmid replication: a promoter operably linked to the gene in question (which may be supplied as the gene's own homologous promoter if it is recognized, i.e., transcribed, by the host), a transcription termination and polyadenylation region (necessary for stability of the mRNA transcribed by the host from the protease gene in certain eucaryotic host cells) which is exogenous or is supplied by the endogenous terminator region of the protease gene and, desirably, a selection gene such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antibiotic-containing media. High copy number plasmids also contain an origin of replication for the host, thereby enabling large numbers of plasmids to be generated in the cytoplasm without chromosomal limitations. However, it is within the scope herein to integrate multiple copies of the protease gene into host genome. This is facilitated by procaryotic and eucaryotic organisms which are particularly susceptible to homologous recombination.

In one embodiment, the gene can be a natural gene such as that from *B lentus* or *B. amyloliquefaciens*. Alternatively, a synthetic gene encoding a naturally-occurring or mutant precursor protease may be produced. In such an approach, the DNA and/or amino acid sequence of the precursor protease is determined. Multiple, overlapping synthetic single-stranded DNA fragments are thereafter synthesized, which upon hybridization and ligation produce a synthetic DNA encoding the precursor protease. An example of synthetic gene construction is set forth in Example 3 of US Patent 5,204,015, the disclosure of which is incorporated herein by reference.

Once the naturally-occurring or synthetic precursor protease gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor protease. Such modifications include the production of recombinant proteases as disclosed in US Patent 4,760,025 (RE 34,606) and EPO Publication No. 0 251 446 and the production of protease variants described herein.

The following cassette mutagenesis method may be used to facilitate the construction of the protease variants of the present invention, although other methods may be used. First, the naturally-occurring gene encoding the protease is obtained and

sequenced in whole or in part. Then the sequence is scanned for a point at which it is desired to make a mutation (deletion, insertion or substitution) of one or more amino acids in the encoded enzyme. The sequences flanking this point are evaluated for the presence of restriction sites for replacing a short segment of the gene with an oligonucleotide pool which when expressed will encode various mutants. Such restriction sites are preferably unique sites within the protease gene so as to facilitate the replacement of the gene segment. However, any convenient restriction site which is not overly redundant in the protease gene may be used, provided the gene fragments generated by restriction digestion can be reassembled in proper sequence. If restriction sites are not present at locations within a convenient distance from the selected point (from 10 to 15 nucleotides), such sites are generated by substituting nucleotides in the gene in such a fashion that neither the reading frame nor the amino acids encoded are changed in the final construction. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by M13 primer extension in accord with generally known methods. The task of locating suitable flanking regions and evaluating the needed changes to arrive at two convenient restriction site sequences is made routine by the redundancy of the genetic code, a restriction enzyme map of the gene and the large number of different restriction enzymes. Note that if a convenient flanking restriction site is available, the above method need be used only in connection with the flanking region which does not contain a site.

Once the naturally-occurring DNA or synthetic DNA is cloned, the restriction sites flanking the positions to be mutated are digested with the cognate restriction enzymes and a plurality of end termini-complementary oligonucleotide cassettes are ligated into the gene. The mutagenesis is simplified by this method because all of the oligonucleotides can be synthesized so as to have the same restriction sites, and no synthetic linkers are necessary to create the restriction sites.

In one aspect of the invention, the objective is to secure a variant protease having altered allergenic potential as compared to the precursor protease, since decreasing such potential enables safer use of the enzyme. While the instant invention is useful to lower allergenic potential, the mutations specified herein may be utilized in combination with mutations known in the art to result altered thermal stability and/or altered substrate specificity, modified activity or altered alkaline stability as compared to the precursor.

Accordingly, the present invention is directed to altering the capability of the T-cell epitope which includes residue positions 170-173 in *Bacillus lentus* to induce T-cell

proliferation. One particularly preferred embodiment of the invention comprises making modification to either one or all of R170D, Y171Q and/or N173D. Similarly, as discussed in detail above, it is believed that the modification of the corresponding residues in any protease will result in a the neutralization of a key T-cell epitope in that protease. Thus, in combination with the presently disclosed mutations in the region corresponding to amino acid residues 170-173, substitutions at positions corresponding to N76D/S103A/V104I/G159D optionally in combination with one or more substitutions selected from the group consisting of positions corresponding to V68A, T213R, A232V, Q236H, Q245R, and T260A of *Bacillus amyloliquefaciens* subtilisin may be used, in addition to decreasing the allergenic potential of the variant protease of the invention, to modulate overall stability and/or proteolytic activity of the enzyme. Similarly, the substitutions provided herein may be combined with mutation at the Asparagine (N) in *Bacillus lentus* subtilisin at equivalent position +76 to Aspartate (D) in combination with the mutations S103A/V104I/G159D and optionally in combination with one or more substitutions selected from the group consisting of positions corresponding to V68A, T213R, A232V, Q236H, Q245R, and T260A of *Bacillus amyloliquefaciens* subtilisin, to produce enhanced stability and/or enhanced activity of the resulting mutant enzyme.

The most preferred embodiments of the invention include the following specific combinations of substituted residues corresponding to positions:
N76D/S103A/V104I/G159D/K170D/Y171Q/S173D;
V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D /Q236H;
V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D /Q236H/Q245R;
V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/A232V/Q236H/Q245R; and
V68A/N76D//S103A/V104I/G159D/K170D/Y171Q/S173D/T213R/A232V/Q236H/
Q245R/T260A of *Bacillus amyloliquefaciens* subtilisin. These substitutions are preferably made in *Bacillus lentus* (recombinant or native-type) subtilisin, although the substitutions may be made in any *Bacillus* protease.

Based on the screening results obtained with the variant proteases, the noted mutations noted above in *Bacillus amyloliquefaciens* subtilisin are important to the proteolytic activity, performance and/or stability of these enzymes and the cleaning or wash performance of such variant enzymes.

Many of the protease variants of the invention are useful in formulating various detergent compositions. A number of known compounds are suitable surfactants useful in compositions comprising the protease mutants of the invention. These include nonionic, anionic, cationic, anionic or zwitterionic detergents, as disclosed in US 4,404,128 to Barry

J. Anderson and US 4,261,868 to Jiri Flora, et al. A suitable detergent formulation is that described in Example 7 of US Patent 5,204,015 (previously incorporated by reference). The art is familiar with the different formulations which can be used as cleaning compositions. In addition to typical cleaning compositions, it is readily understood that the protease variants of the present invention may be used for any purpose that native or wild-type proteases are used. Thus, these variants can be used, for example, in bar or liquid soap applications, dishcare formulations, contact lens cleaning solutions or products, peptide hydrolysis, waste treatment, textile applications, as fusion-cleavage enzymes in protein production, etc. The variants of the present invention may comprise, in addition to decreased allergenicity, enhanced performance in a detergent composition (as compared to the precursor). As used herein, enhanced performance in a detergent is defined as increasing cleaning of certain enzyme sensitive stains such as grass or blood, as determined by usual evaluation after a standard wash cycle.

Proteases of the invention can be formulated into known powdered and liquid detergents having pH between 6.5 and 12.0 at levels of about .01 to about 5% (preferably .1% to .5%) by weight. These detergent cleaning compositions can also include other enzymes such as known proteases, amylases, cellulases, lipases or endoglycosidases, as well as builders and stabilizers.

The addition of proteases of the invention to conventional cleaning compositions does not create any special use limitation. In other words, any temperature and pH suitable for the detergent is also suitable for the present compositions as long as the pH is within the above range, and the temperature is below the described protease's denaturing temperature. In addition, proteases of the invention can be used in a cleaning composition without detergents, again either alone or in combination with builders and stabilizers.

The variant proteases of the present invention can be included in animal feed such as part of animal feed additives as described in, for example, US 5,612,055; US 5,314,692; and US 5,147,642.

One aspect of the invention is a composition for the treatment of a textile that includes variant proteases of the present invention. The composition can be used to treat for example silk or wool as described in publications such as RD 216,034; EP 134,267; US 4,533,359; and EP 344,259.

The following is presented by way of example and is not to be construed as a limitation to the scope of the claims.

The variants can be screened for proteolytic activity according to methods well known in the art. Preferred protease variants include multiple substitutions at positions corresponding to: N76D/S103A/V104I/G159D/K170D/Y171Q/S173D; V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/Q236H; V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/Q236H/Q245R; V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/A232V/Q236H/Q245R; and V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/T213R/A232V/Q236H/Q245R/T260A of *Bacillus amyloliquefaciens* subtilisin.

All publications and patents referenced herein are hereby incorporated by reference in their entirety.

EXAMPLES

Example 1

Assay for the Identification of Peptide T-Cell Epitopes Using Naïve Human T-Cells

Fresh human peripheral blood cells were collected from "naïve" humans, i.e., persons not known to be exposed to or sensitized to *Bacillus lentus* protease, for determination of antigenic epitopes in protease from *Bacillus lentus* and human subtilisin. Naïve humans is intended to mean that the individual is not known to have been exposed to or developed a reaction to protease in the past. Peripheral mononuclear blood cells (stored at room temperature, no older than 24 hours) were prepared for use as follows: Approximately 30 mls of a solution of buffy coat preparation from one unit of whole blood was brought to 50 ml with Dulbecco's phosphate buffered solution (DPBS) and split into two tubes. The samples were underlaid with 12.5 ml of room temperature lymphoprep density separation media (Nycomed density 1.077 g/ml). The tubes were centrifuged for thirty minutes at 600G. The interface of the two phases was collected, pooled and washed in DPBS. The cell density of the resultant solution was measured by hemocytometer. Viability was measured by trypan blue exclusion.

From the resulting solution, a differentiated dendritic cell culture was prepared from the peripheral blood mononuclear cell sample having a density of 10^8 cells per 75 ml culture flask in a solution as follows:

(1) 50 ml of serum free AIM V media (Gibco) was supplemented with a 1:100 dilution beta-mercaptoethanol (Gibco). The flasks were laid flat for two hours at 37°C in 5% CO_2 to allow adherence of monocytes to the flask wall.

(2) Differentiation of the monocyte cells to dendritic cells was as follows: nonadherent cells were removed and the resultant adherent cells (monocytes) combined with 30 ml of AIM V, 800 units/ml of GM-CSF (Endogen) and 500 units/ml of IL-4 (Endogen); the resulting mixture was cultured for 5 days under conditions at 37°C in 5% CO_2. After five days, the cytokine TNFα (Endogen) was added to 0.2 units/ml, and the cytokine IL-1α (Endogen) was added to a final concentration of 50 units/ml and the mixture incubated at 37°C in 5% CO_2 for two more days.

(3) On the seventh day, Mitomycin C was added to a concentration of 50 microgram/ml was added to stop growth of the now differentiated dendritic cell culture. The solution was incubated for 60 minutes at 37°C in 5% CO_2. Dendritic cells were collected by gently scraping the adherent cells off the bottom of the flask with a cell scraper. Adherent and non-adherent cells were then centrifuged at 600G for 5 minutes, washed in DPBS and counted.

(4) The prepared dendritic cells were placed into a 96 well round bottom array at $2x10^4$/well in 100 microliter total volume of AIM V media.

CD4+ T cells were prepared from frozen aliquots of the peripheral blood cell samples used to prepare the dendritic cells using the human CD4+ Cellect Kit (Biotex) as per the manufacturers instructions with the following modifications: the aliquots were thawed and washed such that approximately 10^8 cells will be applied per Cellect column; the cells were resuspended in 4 ml DPBS and 1 ml of the Cell reagent from the Cellect Kit, the solution maintained at room temperature for 20 minutes. The resultant solution was centrifuged for five minutes at 600G at room temperature and the pellet resuspended in 2 ml of DPBS and applied to the Cellect columns. The effluent from the columns was collected in 2% human serum in DPBS. The resultant CD4+ cell solution was centrifuged, resuspended in AIMV media and the density counted.

The CD4+ T-cell suspension was resuspended to a count of $2x10^6$/ml in AIM V media to facilitate efficient manipulation of the 96 well plate.

Peptide antigen is prepared from a 1M stock solution in DMSO by dilution in AIM V media at a 1:10 ratio. 10 microliters of the stock solution is placed in each well of the 96 well plate containing the differentiated dendritic cells. 100 microliter of the diluted CD4+ T-cell solution as prepared above is further added to each well. Useful controls include diluted DMSO blanks, and tetanus toxoid positive controls.

The final concentrations in each well, at 210 microliter total volume are as follows:

$2x10^4$ CD4+

$2x10^5$ dendtritic cells (R:S of 10:1)

5 mM peptide

Example 2

Identification of T-Cell Epitopes in Protease from *Bacillus lentus* and Human subtilisin

Peptides for use in the assay described in Example 1 were prepared based on the *Bacillus lentus* and human subtilisin amino acid sequence. Peptide antigens were designed as follows. From the full length amino acid sequence of either human subtilisin or *Bacillus lentus* protease provided in Figure 1, 15mers were synthetically prepared, each 15mer overlapping with the previous and the subsequent 15mer except for three residues.

Peptides used correspond to amino acid residue strings in *Bacillus lentus* as provided in Figure 8, and peptides correspond to amino acid residues in human subtilisin as provided in Figure 7. The peptides used corresponding to the proteases is provided in Fig. 6. All tests were performed at least in duplicate. All tests reported displayed robust positive control responses to the antigen tetanus toxoid. Responses were averaged within each experiment, then normalized to the baseline response. A positive event was recorded if the response was at least 3 times the baseline response.

The immunogenic response (i.e., T-cell proliferation) to the prepared peptides from human subtilisin and *Bacillus lentus* was tallied and is provided in Figures 4 and 5, respectively. T-cell proliferation was measured by the incorporated tritium method. The results shown in Figures 4 and 5 as a comparison of the immunogenic additive response in 10 individuals (Figure 4) and 16 individuals (Figure 5) to the various peptides. Response is indicated as the added response wherein 1.0 equals a baseline response for each sample. Thus, in Figure 4, a reading of 10.0 or less is the baseline response and in Figure 5 a reading of 16.0 or less the baseline response.

As indicated in Figures 4 and 5, the immunogenic response of the naïve blood samples from unsensitized individuals showed a marked allergenic response at the peptide fragment from *Bacillus lentus* corresponding to residues 170-173 of *Bacillus amyloliquefaciens* protease. As expected, the corresponding fragment in human subtilisin evokes merely baseline response.

Fig. 9 shows the T-cell response to peptides derived from *Bacillus lentus* protease in a sample taken from an individual known to be hypersensitive to *Bacillus lentus*

protease. Peptide E05 represents the region corresponding to 170-173 in protease from *Bacillus amyloliquefaciens.* As shown in Fig. 9, the hypersensitive individual was highly responsive to the T-cell epitope represented by the peptide E05. This result confirms that, by practicing the assay according to the invention, it is possible to predict the major epitopes identified by the T-cells of a hypersensitive individual.

Fig. 10 shows the T-cell response to various alanine substitutions in the E05 peptide derived from *Bacillus lentus* protease in a sample taken from an individual known to be hypersensitive to *Bacillus lentus* protease. Alanine substitutions were used as substitutions for the purpose of determining the role of any specific residue within the epitope. The legend of Figure 10 refers to the position of the peptide in which an alanine was substituted, i.e., in peptide E06 (sequence GSISYPARYANAMAV), G to A = 2, S to A = 3, I to A = 4, S to A = 5, Y to A = 6, P to A = 7, R to A = 8, Y to A = 9, N to A = 10, M to A = 11 and V to A = 12. As indicated in Figure 10, substitution of either of the residues R170A, Y171A and/or N173A in protease from *Bacillus lentus* results in dramatically reduced response in the hypersensitive individual's blood sample.

From these results, it is apparent that the residues 170, 171 and 173 are critical for T-cell response within this peptide. Accordingly, it is further apparent that these residues are largely responsible for the initiation of allergic reaction within the protease from *Bacillus lentus.*

WE CLAIM:

1. A protease variant comprising a substitution made at one or more of positions in a precursor protease corresponding to K170D, Y171Q and/or S173D of *Bacillus amyloliquefaciens* subtilisin.

2. The protease variant according to claim 1, further comprising a substitution at one or more positions in a precursor protease equivalent to those selected from the group consisting of N76D, S103A, V104I, G159D, V68A, T213R, A232V, Q236H, Q245R, and T260A.

3. The protease variant according to claim 2 which is derived from a *Bacillus* subtilisin.

4. The protease variant according to claim 3 which is derived from *Bacillus lentus* subtilisin or *Bacillus amyloliquefaciens* subtilisin.

5. A DNA encoding a protease variant of claim 1.

6. An expression vector encoding the DNA of claim 5.

7. A host cell transformed with the expression vector of claim 6.

8. A cleaning composition comprising the protease variant of claim 1.

9. An animal feed comprising the protease variant of claim 1.

10. A composition for treating a textile comprising the protease variant of claim 1.

11. A protease variant according to claim 1, comprising combined substitution sets selected from the group consisting of positions corresponding to K170D/Y171Q/S173D; N76D/S103A/V104I/G159D/ K170D/Y171Q/S173D; V68A/N76D/S103A/V104I/G159D/ K170D/Y171Q/S173D/Q236H; V68A/N76D/S103A/V104I/G159D/ K170D/Y171Q/S173D/Q236H/Q245R; V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/ A232V/Q236H/Q245R; and V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/T213R/A232V/Q236H/ Q245R/T260A of *Bacillus amyloliquefaciens* subtilisin.

12. A method for determining T-cell epitopes in humans comprising the steps of:

(a) obtaining from a single blood source a solution of dendritic cells and a solution of naïve CD4+ and/or CD8+ T-cells;

(b) promoting differentiation in said solution of dendritic cells;

(c) combining said solution of differentiated dendritic cells and said naïve CD4+ and/or CD8+ T-cells with a peptide of interest;

(d) measuring the production of antibodies in said step (c).

13. A method of reducing the allergenicity of a protein comprising the steps of:

(a) identifying a T-cell epitope in said protein;

(b) modifying said protein to neutralize said T-cell epitope.

14. The method according to claim 13, wherein said epitope is modified by:

(a) substituting the amino acid sequence of the epitope with an analogous sequence from a human homolog to the protein of interest;

(b) substituting the amino acid sequence of the epitope with an analogous sequence from a non-human homolog to the protein of interest, which analogous sequence produces a lesser allergenic response from T-cells than that of the protein of interest; or

(c) substituting the amino acid sequence of the epitope with a sequence which substantially mimics the major tertiary structure attributes of the epitope, but which produces a lesser allergenic response from T-cells than that of the protein of interest.

15. A protein having reduced allergenicity made by the method according to claim 14.

16. A protein having reduced allergenicity, wherein said protein comprises a modification comprising the substitution or deletion of amino acid residues which are identified as within a T-cell epitope according to the assay provided in claim 13.

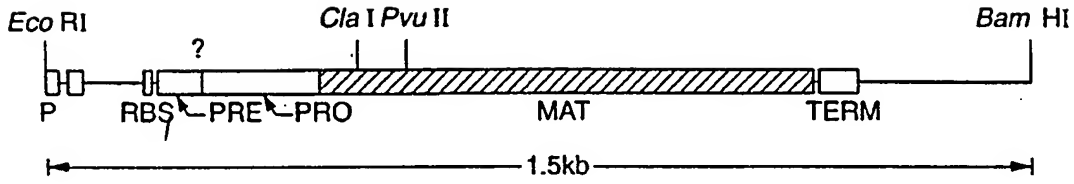
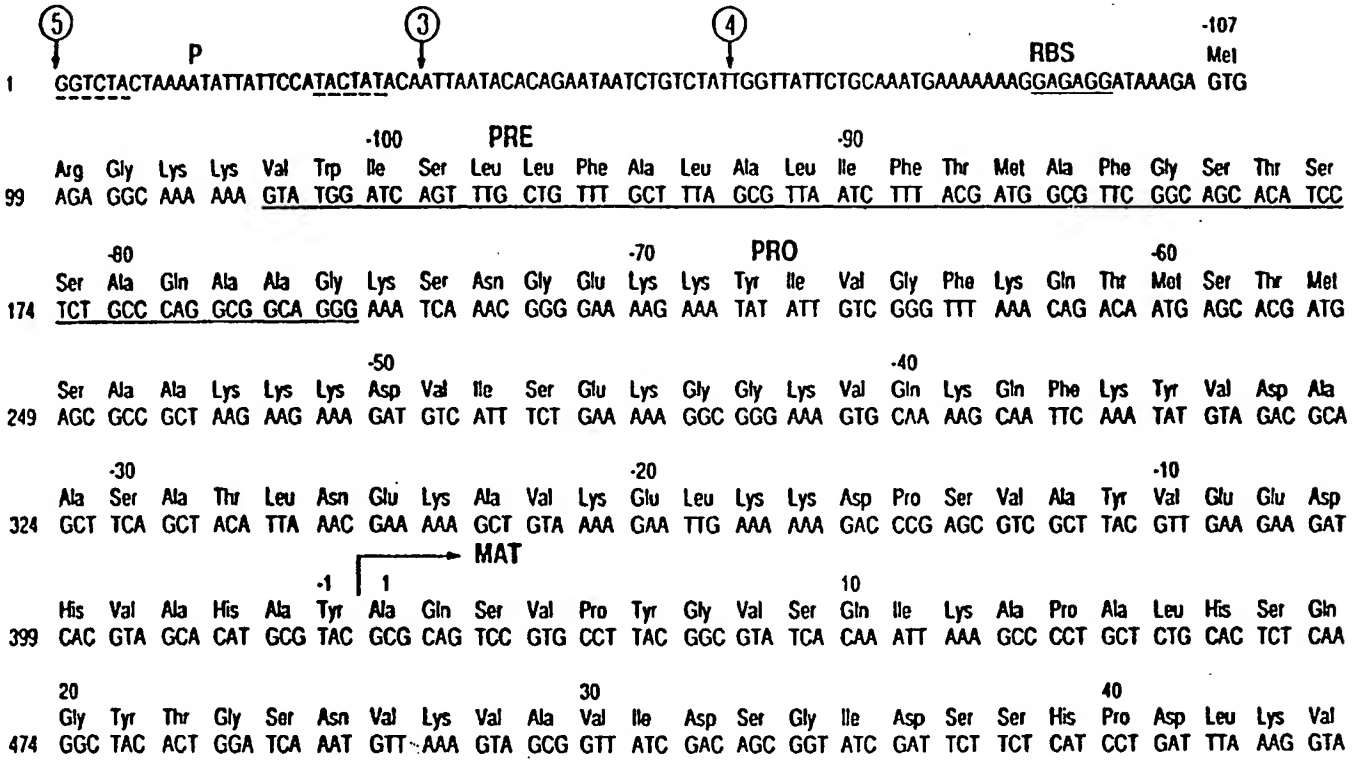


FIG._1A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PRE -100 -90
Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe Ala Leu Ala Leu Ile Phe Thr Met Ala Phe Gly Ser Thr Ser
99 AGA GGC AAA AAA GTA TGG ATC AGT TTG CTG TTT GCT TTA GCG TTA ATC TTT ACG ATG GCG TTC GGC AGC ACA TCC
PRO -80 -70 -60
Ser Ala Gln Ala Ala Gly Lys Ser Asn Gly Glu Lys Lys Tyr Ile Val Gly Phe Lys Gln Thr Met Ser Thr Met
174 TCT GCC CAG GCG GCA GGG AAA TCA AAC GGG GAA AAG AAA TAT ATT GTC GGG TTT AAA CAG ACA ATG AGC ACG ATG
-50 -40
Ser Ala Ala Lys Lys Lys Asp Val Ile Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp Ala
249 AGC GCC GCT AAG AAG AAA GAT GTC ATT TCT GAA AAA GGC GGG AAA GTG CAA AAG CAA TTC AAA TAT GTA GAC GCA
-30 -20 -10
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MAT
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399 CAC GTA GCA CAT GCG TAC GCG CAG TCC GTG CCT TAC GGC GTA TCA CAA ATT AAA GCC CCT GCT CTG CAC TCT CAA
20 30 40
Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val
474 GGC TAC ACT GGA TCA AAT GTT AAA GTA GCG GTT ATC GAC AGC GGT ATC GAT TCT TCT CAT CCT GAT TTA AAG GTA

FIG._1B - 1

						50						Pro	Asn			60	Asp							
	Ala	Gly	Gly	Ala	Ser	Met	Val	Pro	Ser	Glu	Thr	Asn	Pro	Phe	Gln	Asp	Asn	Asn	Ser	His	Gly	Thr	His	Val	Ala
549	GCA	GGC	GGA	GCC	AGC	ATG	GTT	CCT	TCT	GAA	ACA	AAT	CCT	TTC	CAA	GAC	AAC	AAC	TCT	CAC	GGA	ACT	CAC	GTT	GCC
		70									80								Ser	Ala	90				
	Gly	Thr	Val	Ala	Ala	Leu	Asn	Asn	Ser	Ile	Gly	Val	Leu	Gly	Val	Ala	Pro	Ser	Ala	Ser	Leu	Tyr	Ala	Val	Lys
624	GGC	ACA	GTT	GCG	GCT	CTT	AAT	AAC	TCA	ATC	GGT	GTA	TTA	GGC	GTT	GCG	CCA	AGC	GCA	TCA	CTT	TAC	GCT	GTA	AAA
				Asp	Ala	100										110									
	Val	Leu	Gly	Ala	Asp	Gly	Ser	Gly	Gln	Tyr	Ser	Trp	Ile	Ile	Asn	Gly	Ile	Glu	Trp	Ala	Ile	Ala	Asn	Asn	Met
699	GTT	CTC	GGT	GCT	GAC	GGT	TCC	GGC	CAA	TAC	AGC	TGG	ATC	ATT	AAC	GGA	ATC	GAG	TGG	GCG	ATC	GCA	AAC	AAT	ATG
	120										130										140				
	Asp	Val	Ile	Asn	Met	Ser	Leu	Gly	Gly	Pro	Ser	Gly	Ser	Ala	Ala	Leu	Lys	Ala	Ala	Val	Asp	Lys	Ala	Val	Ala
774	GAC	GTT	ATT	AAC	ATG	AGC	CTC	GGC	GGA	CCT	TCT	GGT	TCT	GCT	GCT	TTA	AAA	GCG	GCA	GTT	GAT	AAA	GCC	GTT	GCA
						150								Ser	Thr	160									
	Ser	Gly	Val	Val	Val	Val	Ala	Ala	Ala	Gly	Asn	Glu	Gly	Thr	Ser	Gly	Ser	Ser	Ser	Thr	Val	Gly	Tyr	Pro	Gly
849	TCC	GGC	GTC	GTA	GTC	GTT	GCG	GCA	GCC	GGT	AAC	GAA	GGC	ACT	TCC	GGC	AGC	TCA	AGC	ACA	GTG	GGC	TAC	CCT	GGT
	170										180										190				
	Lys	Tyr	Pro	Ser	Val	Ile	Ala	Val	Gly	Ala	Val	Asp	Ser	Ser	Asn	Gln	Arg	Ala	Ser	Phe	Ser	Ser	Val	Gly	Pro
924	AAA	TAC	CCT	TCT	GTC	ATT	GCA	GTA	GGC	GCT	GTT	GAC	AGC	AGC	AAC	CAA	AGA	GCA	TCT	TTC	TCA	AGC	GTA	GGA	CCT
						200										210									
	Glu	Leu	Asp	Val	Met	Ala	Pro	Gly	Val	Ser	Ile	Gln	Ser	Thr	Leu	Pro	Gly	Asn	Lys	Tyr	Gly	Ala	Tyr	Asn	Gly
999	GAG	CTT	GAT	GTC	ATG	GCA	CCT	GGC	GTA	TCT	ATC	CAA	AGC	ACG	CTT	CCT	GGA	AAC	AAA	TAC	GGG	GCG	TAC	AAC	GGT
	220										230										240				
	Thr	Ser	Met	Ala	Ser	Pro	His	Val	Ala	Gly	Ala	Ala	Ala	Leu	Ile	Leu	Ser	Lys	His	Pro	Asn	Trp	Thr	Asn	Thr
1074	ACG	TCA	ATG	GCA	TCT	CCG	CAC	GTT	GCC	GGA	GCG	GCT	GCT	TTG	ATT	CTT	TCT	AAG	CAC	CCG	AAC	TGG	ACA	AAC	ACT

FIG._1B - 2

```
                                    250 Gln                                                 260
     Gln  Val  Arg  Ser  Ser  Leu  Glu  Asn  Thr  Thr  Thr  Lys  Leu  Gly  Asp  Ser  Phe  Tyr  Tyr  Gly  Lys  Gly  Leu  Ile  Asn
1149 CAA  GTC  CGC  AGC  AGT  TTA  GAA  AAC  ACC  ACT  ACA  AAA  CTT  GGT  GAT  TCT  TTC  TAC  TAT  GGA  AAA  GGG  CTG  ATC  AAC

     270                      275
     Val  Gln  Ala  Ala  Ala  Gln  OC                    TERM
1224 GTA  CAG  GCG  GCA  GCT  CAG  TAA  AACATAAAAAACCGGCCTTGGCCCCGCCGGTTTTTTATTTTTCTTCCTCCGCATGTTCAATCCGCTCC

1316 ATAATCGACGGATGGCTCCCTCTGAAAATTTTAACGAGAAACGGCGGGTTGACCCGGCTCAGTCCCGTAACGGCCAAGTCCTGAAACGTCTCAATCGCCG

1416 CTTCCCGGTTTCCGGTCAGCTCAATGCCGTAACGGTCGGCGGCGTTTTCCTGATACCGGGAGACGGCATTCGTAATCGGATC
```

FIG._1B - 3

FIG._1B

- *FIG._1B - 1*
- *FIG._1B - 2*
- *FIG._1B - 3*

CONSERVED RESIDUES IN SUBTILISINS FROM
BACILLUS AMYLOLIQUEFACIENS

```
1                   10                  20
A Q S V P . G . . . . . A P A . H . . G

21                  30                  40
. T G S . V K V A V . D . G . . . . H P

41                  50                  60
D L . . . G G A S . V P . . . . . Q D

61                  70                  80
. N . H G T H V A G T . A A L N N S I G

81                  90                  100
V L G V A P S A . L Y A V K V L G A . G

101                 110                 120
S G . . S . L . . G . E W A . N . . . .

121                 130                 140
V . N . S L G . P S . S . . . . . A . .

141                 150                 160
. . . . . G V . V V A A . G N . G . . .

161                 170                 180
. . . . . Y P . . Y . . . . A V G A .

181                 190                 200
D . . N . . A S F S . . G . . L D . . A

201                 210                 220
P G V . . Q S T . P G . . Y . . . N G T

221                 230                 240
S M A . P H V A G A A A L . . . K . . .

241                 250                 260
W . . . Q . R . . L . N T . . . L G . .

261                 270
. . Y G . G L . N . . A A . .
```

FIG._2

COMPARISON OF SUBTILISIN SEQUENCES FROM:
B.amyloliquefaciens
B.subtilis
B.licheniformis
B.lentus

```
01                  10                  20                  30
A Q S V P Y G V S Q I K A P A L H S Q G Y T G S N V K V A V I D S G I D S S H P
A Q S V P Y G I S Q I K A P A L H S Q G Y T G S N V K V A V I D S G I D S S H P
A Q T V P Y G I P L I K A D K V Q A Q G F K G A N V K V A V L D T G I Q A S H P
A Q S V P W G I S R V Q A P A A H N R G L T G S G V K V A V L D T G I S T * H P

41                  50                  60                  70
D L K V A G G A S M V P S E T N P F Q D N N S H G T H V A G T V A A L N N S I G
D L N V R G G A S F V P S E T N P Y Q D G S S H G T H V A G T I A A L N N S I G
D L N V V G G A S F V A G E A Y N * T D G N G H G T H V A G T V A A L D N T T G
D L N I R G G A S F V P G E * P S T Q D G N G H G T H V A G T I A A L N N S I G

81                  90                  100                 110
V L G V A P S A S L Y A V K V L G A D G S G Q Y S W I I N G I E W A I A N N M D
V L G V S P S A S L Y A V K V L D S T G S G Q Y S W I I N G I E W A I S N N M D
V L G V A P S V S L Y A V K V L N S S G S G S Y S G I V S G I E W A T T N G M D
V L G V A P S A E L Y A V K V L G A S G S G S V S S I A Q G L E W A G N N G M H

121                 130                 140                 150
V I N M S L G G P S G S A A L K A A V D K A V A S G V V V V A A A G N E G T S G
V I N M S L G G P T G S T A L K T V V D K A V S S G I V V A A A A G N E G S S G
V I N M S L G G A S G S T A M K Q A V D N A Y A R G V V V V A A A G N S G N S G
V A N L S L G S P S P S A T L E Q A V N S A T S R G V L V V A A S G N S G A G S
```

FIG._3A

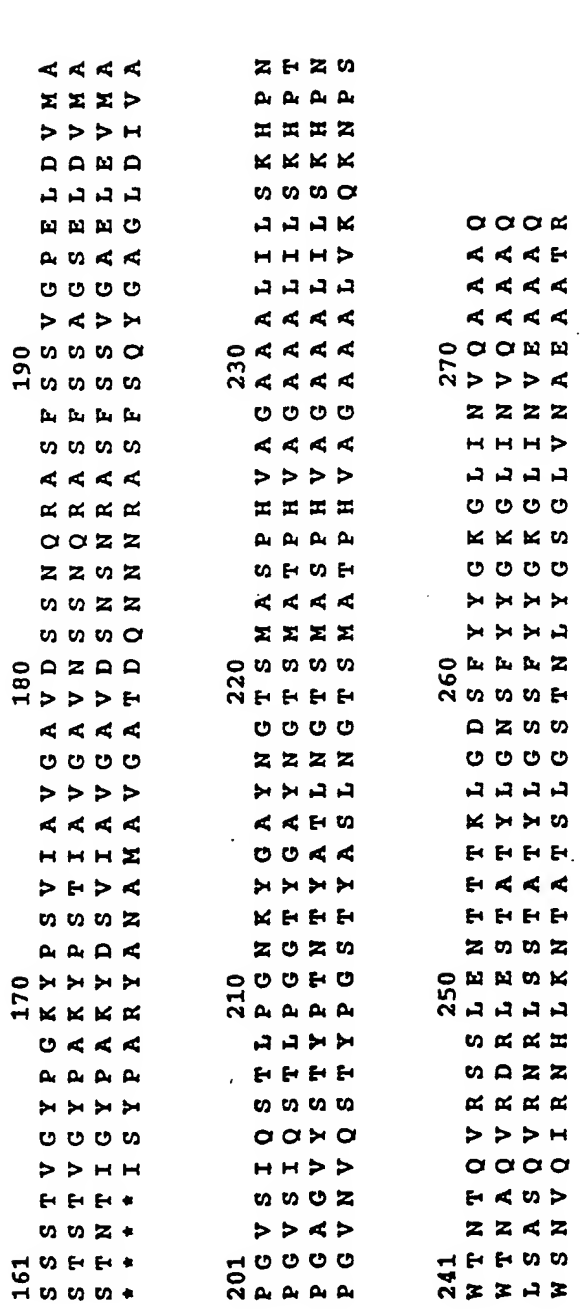

FIG._3B

FIG._3

FIG._3A

FIG._3B

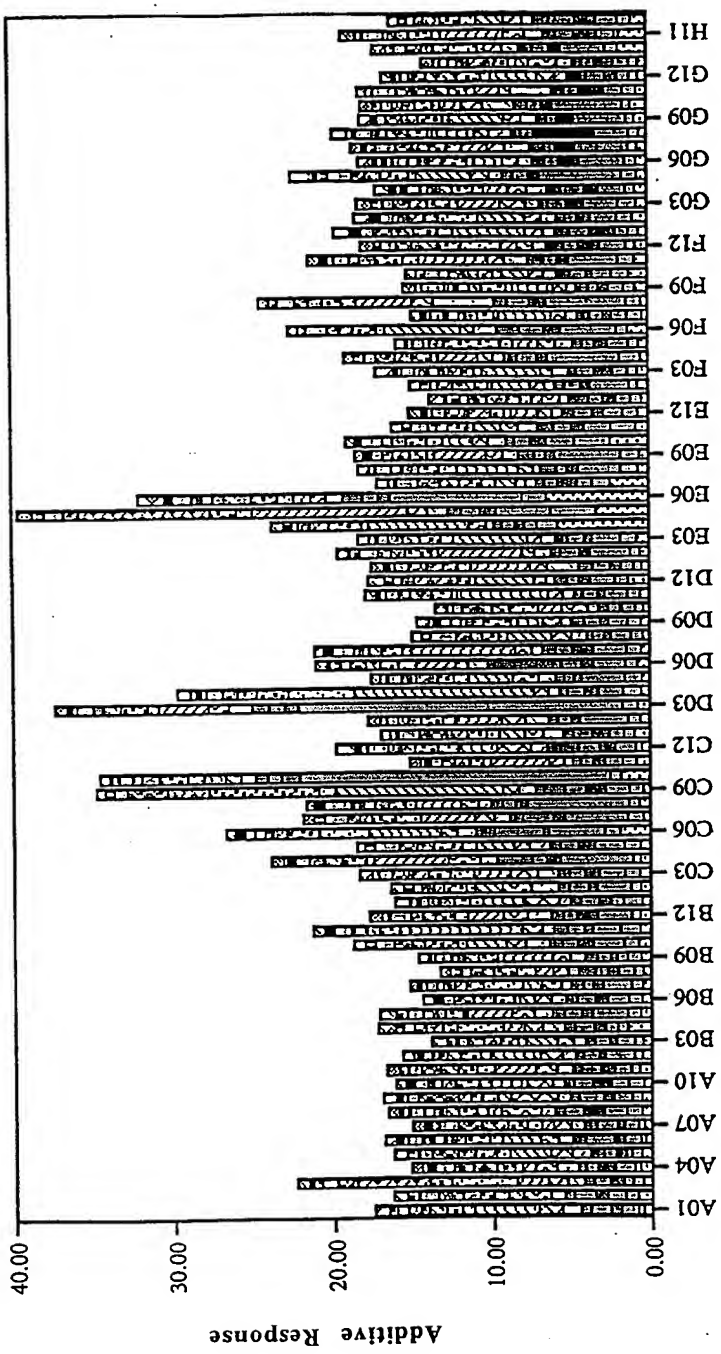
H11
G12
G09
G06
G03
F12
F09
F06
F03
E12
E09
E06
E03
D12
D09
D06
D03
C12
C09
C06
C03
B12
B09
B06
B03
A10
A07
A04
A01
40.00
30.00
20.00
10.00
0.00
Additive Response

FIG. 4

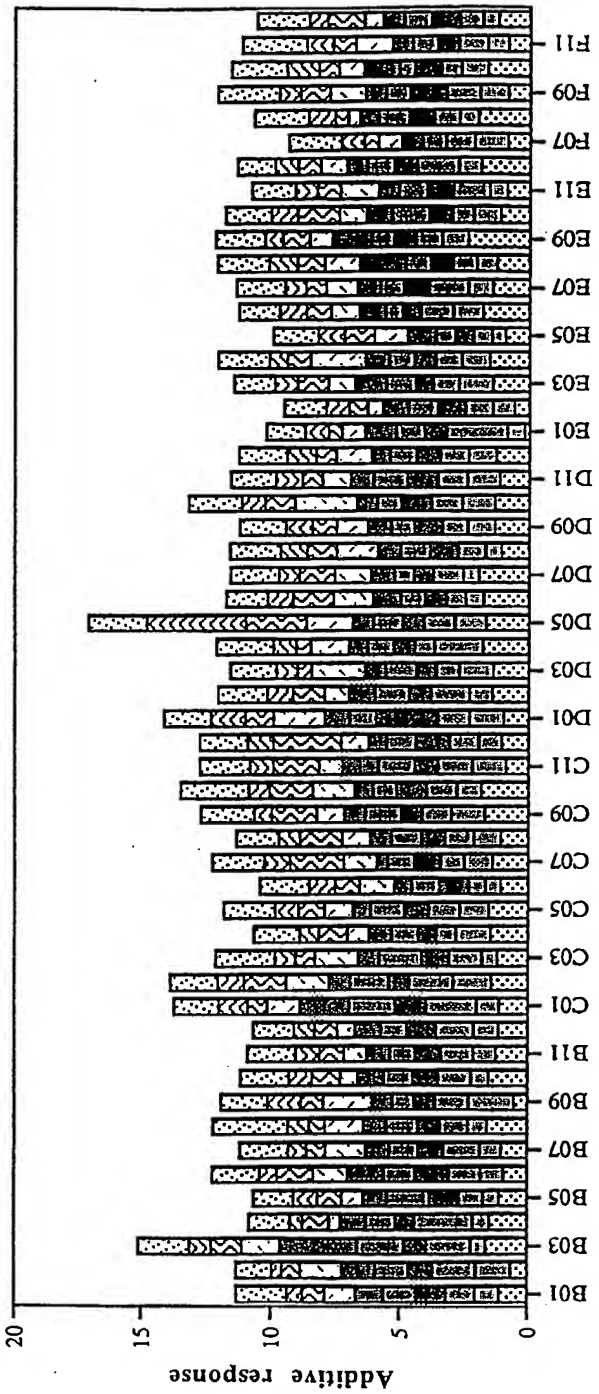
Additive response
0
5
10
15
20
B01
B03
B05
B07
B09
B11
C01
C03
C05
C07
C09
C11
D01
D03
D05
D07
D09
D11
E01
E03
E05
E07
E09
E11
F07
F09
F11

FIG. 5

1	A12	IKDFHVYFRESRDAG
2	A11	LEQAVNSATSRGVLV
3	A10	AQSVPWGISRVQAPA
4	A9	VPWGISRVQAPAAHN
5	A8	GISRVQAPAAHNRGL
6	A7	RVQAPAAHNRGLTGS
7	A6	APAAHNRGLTGSGVK
8	A5	AHNRGLTGSGVKVAV
9	A4	RGLTGSGVKVAVLDT
10	A3	TGSGVKVAVLDTGIS
11	A2	GVKVAVLDTGISTHP
12	A1	VAVLDTGISTHPDLN
13	B12	LDTGISTHPDLNIRG
14	B11	GISTHPDLNIRGGAS
15	B10	THPDLNIRGGASFVP
16	B9	DLNIRGGASFVPGEP
17	B8	IRGGASFVPGEPSTQ
18	B7	GASFVPGEPSTQDGN
19	B6	FVPGEPSTQDGNGHG
20	B5	GEPSTQDGNGHGTHV
21	B4	STQDGNGHGTHVAGT
22	B3	DGNGHGTHVAGTIAA
23	B2	GHGTHVAGTIAALNN
24	B1	THVAGTIAALNNSIG
25	C12	AGTIAALNNSIGVLG
26	C11	IAALNNSIGVLGVAP
27	C10	LNNSIGVLGVAPSAE
28	C9	SIGVLGVAPSAELYA
29	C8	VLGVAPSAELYAVKV
30	C7	VAPSAELYAVKVLGA
31	C6	SAELYAVKVLGASGS
32	C5	LYAVKVLGASGSGSV
33	C4	VKVLGASGSGSVSSI
34	C3	LGASGSGSVSSIAQG
35	C2	SGSGSVSSIAQGLEW
36	C1	GSVSSIAQGLEWAGN
37	D12	SSIAQGLEWAGNNGM
38	D11	AQGLEWAGNNGMHVA
39	D10	LEWAGNNGMHVANLS
40	D9	AGNNGMHVANLSLGS
41	D8	NGMHVANLSLGSPSP
42	D7	HVANLSLGSPSPSAT
43	D6	NLSLGSPSPSATLEQ
44	D5	LGSPSPSATLEQAVN
45	D4	PSPSATLEQAVNSAT
46	D3	SATLEQAVNSATSRG
47	D2	LEQAVNSATSRGVLV
48	D1	AVNSATSRGVLVVAA
49	E12	SATSRGVLVVAASGN
50	E11	SRGVLVVAASGNSGA
51	E10	VLVVAASGNSGAGSI
52	E9	VAASGNSGAGSISYP
53	E8	SGNSGAGSISYPARY
54	E7	SGAGSISYPARYANA
55	E6	GSISYPARYANAMAV
56	E5	SYPARYANAMAVGAT
57	E4	ARYANAMAVGATDQN
58	E3	ANAMAVGATDQNNNR
59	E2	MAVGATDQNNNRASF
60	E1	GATDQNNNRASFSQY
61	F12	DQNNNRASFSQYGAG
62	F11	NNRASFSQYGAGLDI
63	F10	ASFSQYGAGLDIVAP
64	F9	SQYGAGLDIVAPGVN
65	F8	GAGLDIVAPGVNVQS
66	F7	LDIVAPGVNVQSTYP
67	F6	VAPGVNVQSTYPGST
68	F5	GVNVQSTYPGSTYAS
69	F4	VQSTYPGSTYASLNG
70	F3	TYPGSTYASLNGTSM
71	F2	GSTYASLNGTSMATP
72	F1	YASLNGTSMATPHVA
73	G12	LNGTSMATPHVAGAA
74	G11	TSMATPHVAGAAALV
75	G10	ATPHVAGAAALVKQK
76	G9	HVAGAAALVKQKNPS
77	G8	GAAALVKQKNPSWSN
78	G7	ALVKQKNPSWSNVQI
79	G6	KQKNPSWSNVQIRNH
80	G5	NPSWSNVQIRNHLKN
81	G4	WSNVQIRNHLKNTAT
82	G3	VQIRNHLKNTATSLG
83	G2	RNHLKNTATSLGSTN
84	G1	LKNTATSLGSTNLYG
85	H12	TATSLGSTNLYGSGL
86	H11	SLGSTNLYGSGLVNA
87	H10	STNLYGSGLVNAEAA
88	H9	NLYGSGLVNAEAATR

FIG. 6A

1	A12	IKDFHVYFRESRDAG	49	E12	KKIDVLNLSIGGPDF
2	A11	DAELHIFRVFTNNQV	50	E11	DVLNLSIGGPDFMDH
3	A10	PLRRASLSLGSGFWH	51	E10	NLSIGGPDFMDHPFV
4	A9	RASLSLGSGFWHATG	52	E9	IGGPDFMDHPFVDKV
5	A8	LSLGSGFWHATGRHS	53	E8	PDFMDHPFVDKVWEL
6	A7	GSGFWHATGRHSSRR	54	E7	MDHPFVDKVWELTAN
7	A6	FWHATGRHSSRRLLR	55	E6	PFVDKVWELTANNVI
8	A5	ATGRHSSRRLLRAIP	56	E5	DKVWELTANNVIMVS
9	A4	RHSSRRLLRAIPRQV	57	E4	WELTANNVIMVSAIG
10	A3	SRRLLRAIPRQVAQT	58	E3	TANNVIMVSAIGNDG
11	A2	LLRAIPRQVAQTLQA	59	E2	NVIMVSAIGNDGPLY
12	A1	AIPRQVAQTLQADVL	60	E1	MVSAIGNDGPLYGTJ
13	B12	RQVAQTLQADVLWQM	61	F12	AIGNDGPLYGTLNNP
14	B11	AQTLQADVLWQMGYT	62	F11	NDGPLYGTLNNPADQ
15	B10	LQADVLWQMGYTGAN	63	F10	PLYGTLNNPADQMDV
16	B9	DVLWQMGYTGANVRV	64	F9	GTLNNPADQMDVIGV
17	B8	WQMGYTGANVRVAVF	65	F8	NNPADQMDVIGVGGI
18	B7	GYTGANVRVAVFDTG	66	F7	ADQMDVIGVGGIDFE
19	B6	GANVRVAVFDTGLSE	67	F6	MDVIGVGGIDFEDNI
20	B5	VRVAVFDTGLSEKHP	68	F5	IGVGGIDFEDNIARF
21	B4	AVFDTGLSEKHPHFK	69	F4	GGIDFEDNIARFSSR
22	B3	DTGLSEKHPHFKNVK	70	F3	DFEDNIARFSSRGMT
23	B2	LSEKHPHFKNVKERT	71	F2	DNIARFSSRGMTTWE
24	B1	KHPHFKNVKERTNWT	72	F1	ARFSSRGMTTWELPG
25	C12	HFKNVKERTNWTNER	73	G12	SSRGMTTWELPGGYG
26	C11	NVKERTNWTNERTLD	74	G11	GMTTWELPGGYGRMK
27	C10	ERTNWTNERTLDDGL	75	G10	TWELPGGYGRMKPDI
28	C9	NWTNERTLDDGLGHG	76	G9	LPGGYGRMKPDIVTY
29	C8	NERTLDDGLGHGTFV	77	G8	GYGRMKPDIVTYGAG
30	C7	TLDDGLGHGTFVAGV	78	G7	RMKPDIVTYGAGVRG
31	C6	DGLGHGTFVAGVIAS	79	G6	PDIVTYGAGVRGSGV
32	C5	GHGTFVAGVIASMRE	80	G5	VTYGAGVRGSGVKGG
33	C4	TFVAGVIASMRECQG	81	G4	GAGVRGSGVKGGCRA
34	C3	AGVIASMRECQGFAP	82	G3	VRGSGVKGGCRALSG
35	C2	IASMRECQGFAPDAE	83	G2	SGVKGGCRALSGTSV
36	C1	MRECQGFAPDAELHI	84	G1	KGGCRALSGTSVASP
37	D12	CQGFAPDAELHIFRV	85	H12	CRALSGTSVASPVVA
38	D11	FAPDAELHIFRVFTN	86	H11	LSGTSVASPVVAGAV
39	D10	DAELHIFRVFTNNQV	87	H10	TSVASPVVAGAVTLL
40	D9	LHIFRVFTNNQVSYT	88	H9	ASPVVAGAVTLLVST
41	D8	FRVFTNNQVSYTSWF	89	H8	VVAGAVTLLVSTVQK
42	D7	FTNNQVSYTSWFLDA	90	H7	GAVTLLVSTVQKREL
43	D6	NQVSYTSWFLDAFNY	91	H6	TLLVSTVQKRELVNP
44	D5	SYTSWFLDAFNYAIL	92	H5	VSTVQKRELVNPASM
45	D4	SWFLDAFNYAILKKI	93	H4	VQKRELVNPASMKQA
46	D3	LDAFNYAILKKIDVL	94	H3	RELVNPASMKQALIA
47	D2	FNYAILKKIDVLNLS	95	H2	VNPASMKQALIASAR
48	D1	AILKKIDVLNLSIGG	96	H1	ASMKQALIASARRLP

FIG. 6B

97	I12	IKDFHVYFRESRDAG
98	I11	DAELHIFRVFTNNQV
99	I10	KQALIASARRLPGVN
100	I9	LIASARRLPGVNMFE
101	I8	SARRLPGVNMFEQGH
102	I7	RLPGVNMFEQGHGKL
103	I6	GVNMFEQGHGKLDLL
104	I5	MFEQGHGKLDLLRAY
105	I4	QGHGKLDLLRAYQIL
106	I3	GKLDLLRAYQILNSY
107	I2	DLLRAYQILNSYKPQ
108	I1	RAYQILNSYKPQASL
109	J12	QILNSYKPQASLSPS
110	J11	NSYKPQASLSPSYID
111	J10	KPQASLSPSYIDLTE
112	J9	ASLSPSYIDLTECPY
113	J8	SPSYIDLTECPYMWP
114	J7	YIDLTECPYMWPYCS
115	J6	LTECPYMWPYCSQPI
116	J5	CPYMWPYCSQPIYYG

FIG. 6C

MKLVNIWLLLLVVLLCGKKHLGDRLEKKSFEKAPCPGCSHLTLKVEFSSTVVEYEYIVAFNGYFT
AKARNSFISSALKSSEVDNWRIIPRNNPSSDYPSDFEVIQIKEKQKAGLLTLEDHPNIKRVTPQR
KVFRSLKYAESDPTVPCNETRWSQKWQSSRPLRRASLSLGSGFWHATGRHSSRRLLRAIPRQVAQ
TLQADVLWQMGYTGANVRVAVFDTGLSEKHPHFKNVKERTNWTNERTLDDGLGHGTFVAGVIASM
RECQGFAPDAELHIFRVFTNNQVSYTSWFLDAFNYAILKKIDVLNLSIGGPDFMDHPFVDKVWEL
TANNVIMVSAIGNDGPLYGTLNNPADQMDVIGVGGIDFEDNIARFSSRGMTTWELPGGYGRMKPD
IVTYGAGVRGSGVKGGCRALSGTSVASPVVAGAVTLLVSTVQKRELVNPASMKQALIASARRLPG
VNMFEQGHGKLDLLRAYQILNSYKPQASLSPSYIDLTECPYMWPYCSQPIYYGGMPTVVNVTILN
GMGVTGRIVDKPDWQPYLPQNGDNIEVAFSYSSVLWPWSGYLAISISVTKKAASWEGIAQGHVMI
TVASPAETESKNGAEQTSTVKLPIKVKIIPTPPRSKRVLWDQYHNLRYPPGYFPRDNLRMKNDPL
DWNGDHIHTNFRDMYQHLRSMGYFVEVLGAPFTCFDASQYGTLLMVDSEEEYFPEEIAKLRRDVD
NGLSLVIFSDWYNTSVMRKVKFYDENTRQWWMPDTGGANIPALNELLSVWNMGFSDGLYEGEFTL
ANHDMYYASGCSIAKFPEDGVVITQTFKDQGLEVLKQETAVVENVPILGLYQIPAEGGGRIVLYG
DSNCLDDSHRQKDCFWLLDALLQYTSYGVTPPSLSHSGNRQRPPSGAGSVTPERMEGNHLHRYSK
VLEAHLGDPKPRPLPACPRLSWAKPQPLNETAPSNLWKHQKLLSIDLDKVVLPNFRSNRPQVRPL
SPGESGAWDIPGGIMPGRYNQEVGQTIPVFAFLGAMVVLAFFVVQINKAKSRPKRRKPRVKRPQL
MQQVHPPKTPSV

FIG. 7

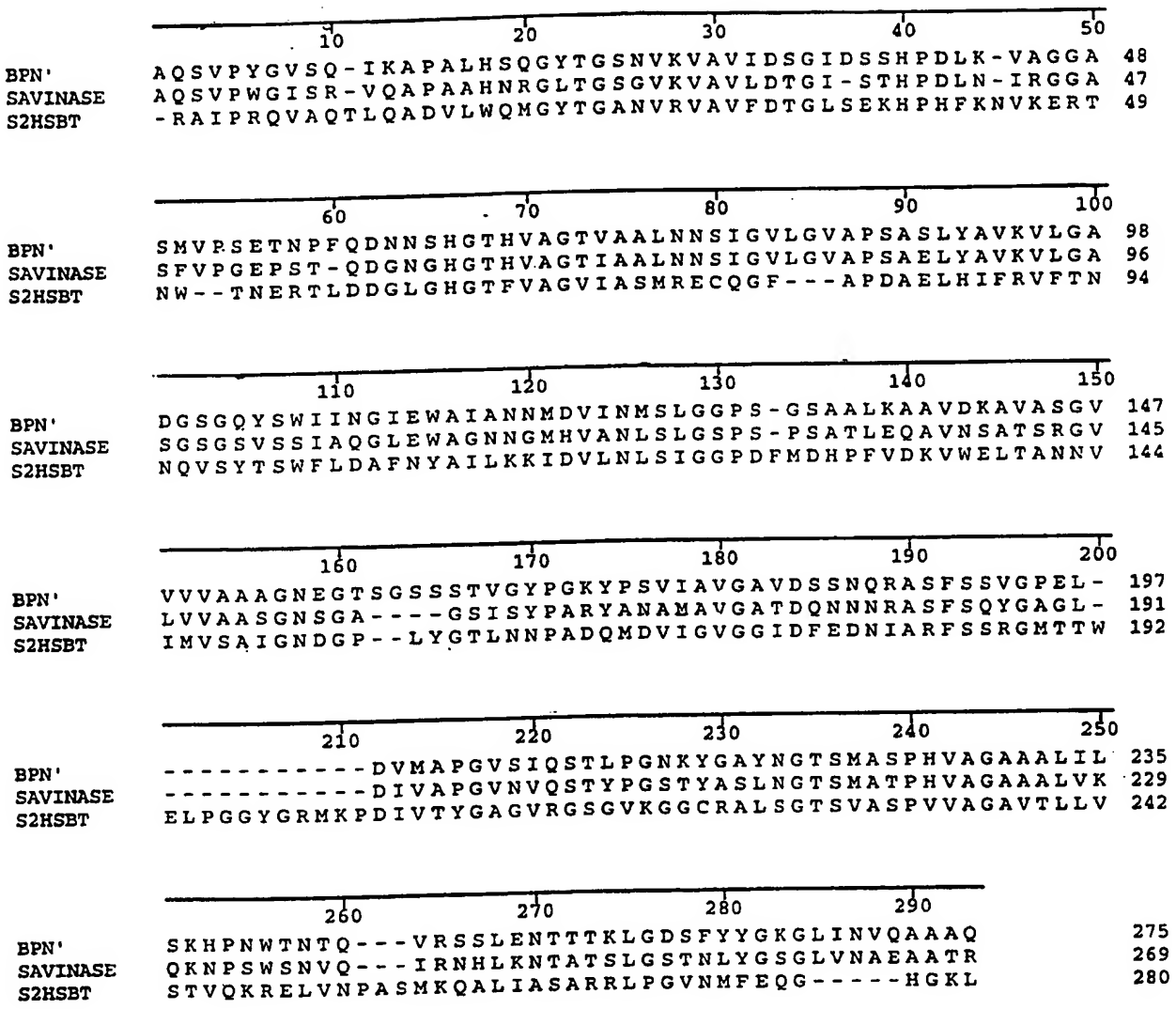
10 20 30 40 50
BPN' AQSVPYGVSQ-IKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLK-VAGGA 48
SAVINASE AQSVPWGISR-VQAPAAHNRGLTGSGVKVAVLDTGI-STHPDLN-IRGGA 47
S2HSBT -RAIPRQVAQTLQADVLWQMGYTGANVRVAVFDTGLSEKHPHFKNVKERT 49
60 70 80 90 100
BPN' SMVPSETNPFQDNNSHGTHVAGTVAALNNSIGVLGVAPSASLYAVKVLGA 98
SAVINASE SFVPGEPST-QDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGA 96
S2HSBT NW--TNERTLDDGLGHGTFVAGVIASMRECQGF---APDAELHIFRVFTN 94
110 120 130 140 150
BPN' DGSGQYSWIINGIEWAIANNMDVINMSLGGPS-GSAALKAAVDKAVASGV 147
SAVINASE SGSGSVSSIAQGLEWAGNNGMHVANLSLGSPS-PSATLEQAVNSATSRGV 145
S2HSBT NQVSYTSWFLDAFNYAILKKIDVLNLSIGGPDFMDHPFVDKVWELTANNV 144
160 170 180 190 200
BPN' VVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFSSVGPEL- 197
SAVINASE LVVAASGNSGA----GSISYPARYANAMAVGATDQNNNRASFSQYGAGL- 191
S2HSBT IMVSAIGNDGP--LYGTLNNPADQMDVIGVGGIDFEDNIARFSSRGMTTW 192
210 220 230 240 250
BPN' ----------DVMAPGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALIL 235
SAVINASE ----------DIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVK 229
S2HSBT ELPGGYGRMKPDIVTYGAGVRGSGVKGGCRALSGTSVASPVVAGAVTLLV 242
260 270 280 290
BPN' SKHPNWTNTQ---VRSSLENTTTKLGDSFYYGKGLINVQAAAQ 275
SAVINASE QKNPSWSNVQ---IRNHLKNTATSLGSTNLYGSGLVNAEAATR 269
S2HSBT STVQKRELVNPASMKQALIASARRLPGVNMFEQG-----HGKL 280

FIG. 8

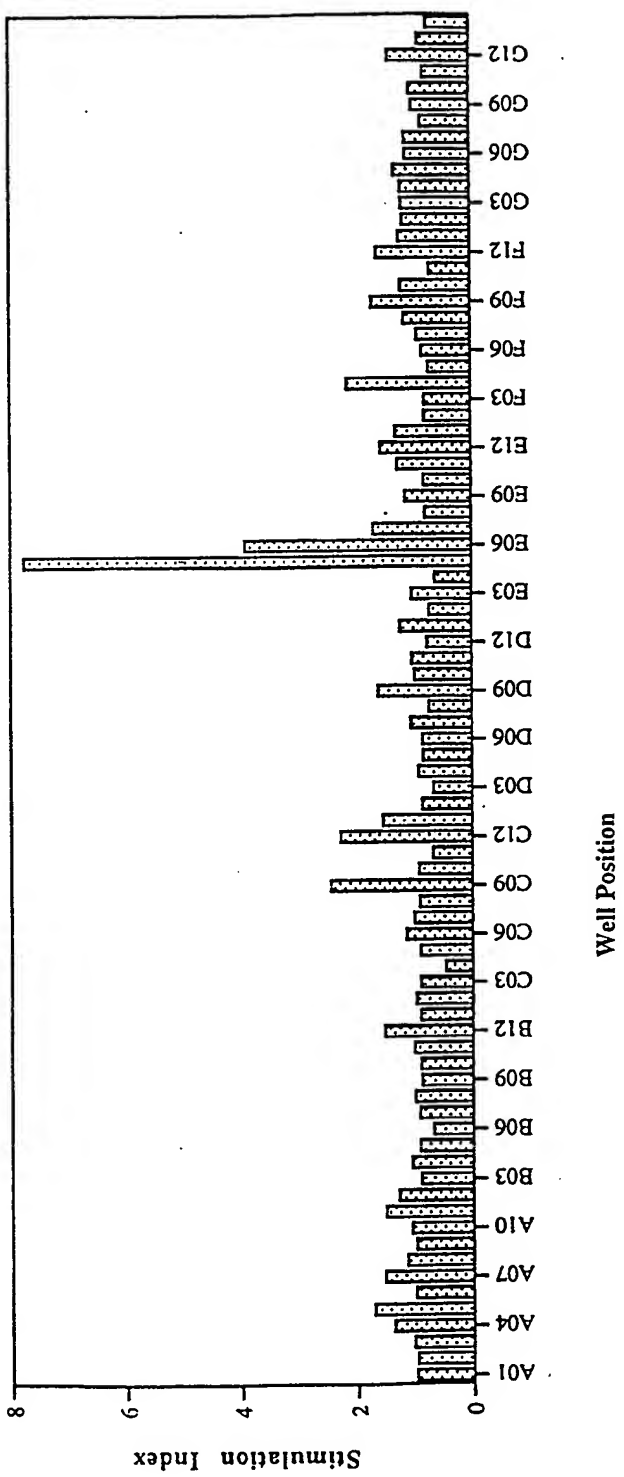
Stimulation Index
0
2
4
6
8
A01
A04
A07
A10
B03
B06
B09
B12
C03
C06
C09
C12
D03
D06
D09
D12
E03
E06
E09
E12
F03
F06
F09
F12
G03
G06
G09
G12
Well Position

FIG. 9

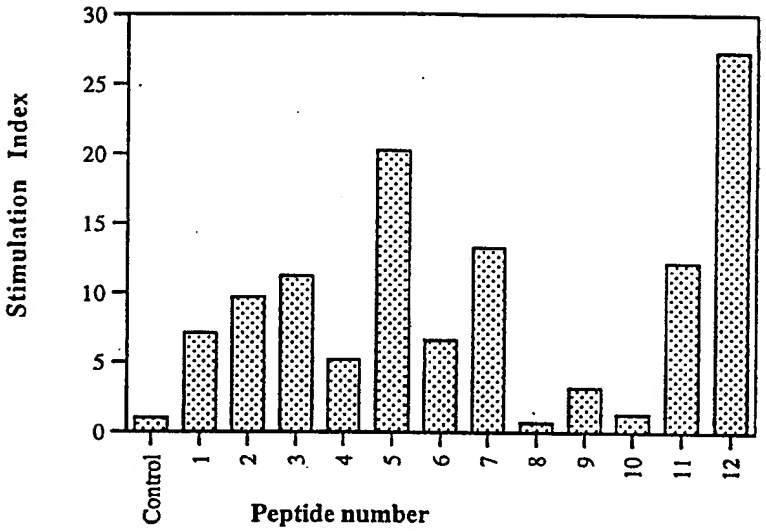
Stimulation Index
30
25
20
15
10
5
0
Control
1
2
3
4
5
6
7
8
9
10
11
12
Peptide number

FIG. 10

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(54) Title: MUTANT PROTEINS HAVING LOWER ALLERGENIC RESPONSE IN HUMANS AND METHODS FOR CONSTRUCTING, IDENTIFYING AND PRODUCING SUCH PROTEINS

(57) Abstract

The present invention relates to a novel improved protein mutant which produces low allergenic response in humans compared to the parent of that mutant. Specifically, the present invention comprises neutralizing or reducing the ability of T–cells to recognize epitopes and thus prevent sensitization of an individual to the protein.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/08253

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/57 C12N15/63 C12N9/54 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 98 20116 A (NOVONORDISK AS) 14 May 1998 (1998-05-14) * see claims 13-17* the whole document	1-8

X	WO 96 34946 A (NOVONORDISK AS) 7 November 1996 (1996-11-07) * see claims 13-15 * the whole document	1-11

Y	WO 92 10755 A (NOVONORDISK AS) 25 June 1992 (1992-06-25)	1-11
X	the whole document	13,14

Y	EP 0 006 638 A (NOVO INDUSTRI AS) 9 January 1980 (1980-01-09) the whole document	1-11

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 November 1999	10. 12. 99

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hillenbrand, G

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/08253

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GUNDLACH B R ET AL: "Determination of T cell epitopes with random peptide libraries" JOURNAL OF IMMUNOLOGICAL METHODS,NL,ELSEVIER SCIENCE PUBLISHERS B.V.,AMSTERDAM, vol. 192, no. 1, page 149-155 XP004020829 ISSN: 0022-1759 abstract	12
Y	WO 97 30148 A (NOVONORDISK AS ;PRENTOE ANNETTE (DK); BISGAARD FRANTZEN HENRIK (DK) 21 August 1997 (1997-08-21) the whole document	13
Y	WO 96 16177 A (NOVONORDISK AS ;BJOERNVAD MADS ESKELUND (DK); PRENTOE ANNETTE (DK)) 30 May 1996 (1996-05-30) the whole document	13

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/08253

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 15-16
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

The subject-matter of claims 15-16 is so broadly and imprecisely drafted with respect to the claimed proteins that no meaningful search could be carried out.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99/08253

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 15-16

The subject-matter of claims 15-16 is so broadly and imprecisely drafted with respect to the claimed proteins that no meaningful search could be carried out.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99/08253

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-11

   The subject-matter of claims 1-11 is directed to specific protease (subtilisin) variants, a DNA encoding said variants, an expression vector encoding said DNA, and a cleaning composition or animal feed comprising said protease (subtilisin) variants.

2. Claim : 12

   The subject-matter of claim 12 is directed to a method for determining T-cell epitopes in humans.

3. Claims: 13-14

   The subject-matter of claims 13-14 is directed to a method of reducing the allergenicity of a protein.

The subject-matter of the present set of claims is not so linked together by a specific technical feature as to form a single general inventive concept.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/08253

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